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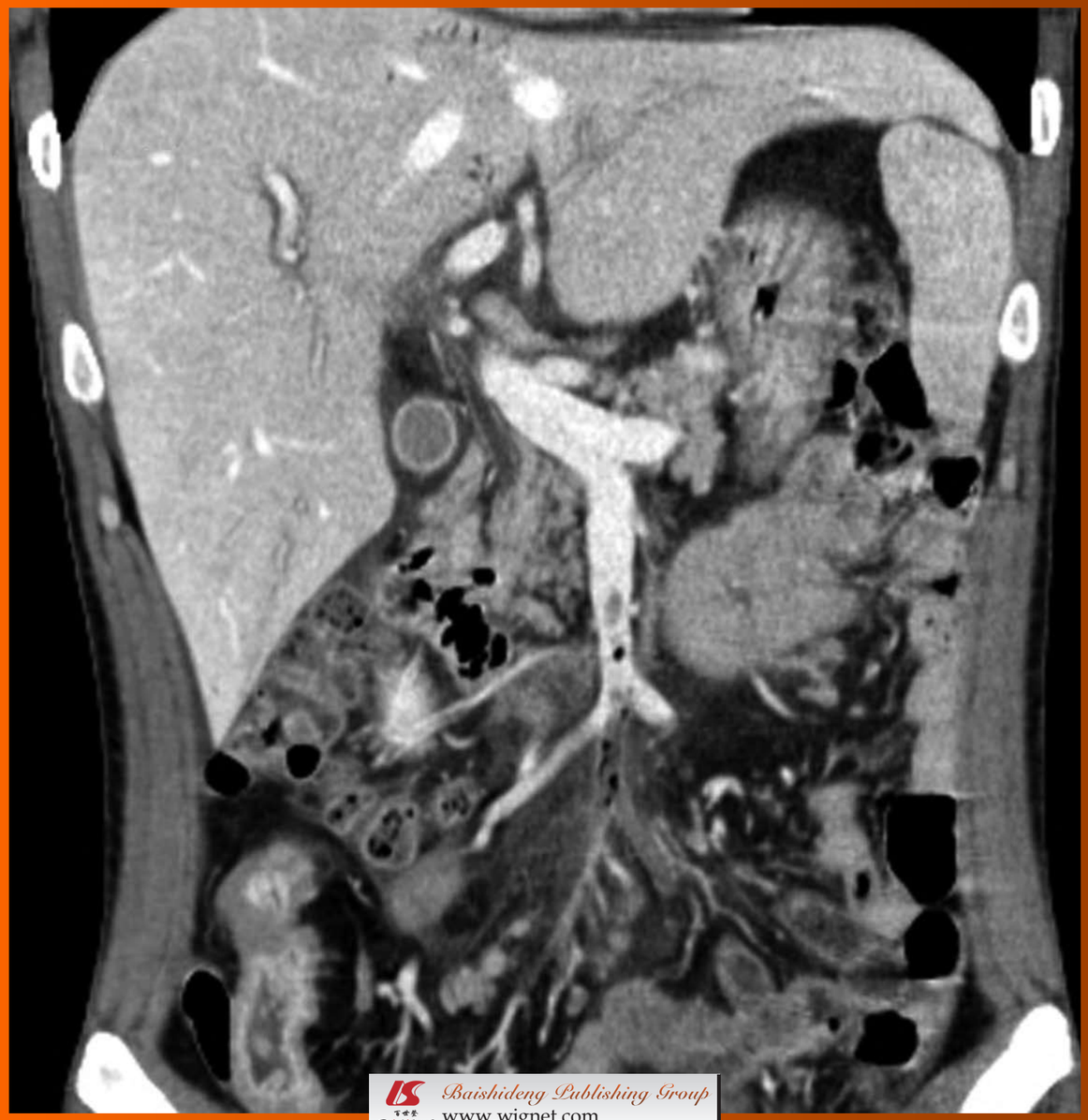

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EDITORIAL

In vivo magnetic resonance spectroscopy of liver tumors and metastases

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Abstract

Primary liver cancer is the fifth most common malignancy in men and the eighth in women worldwide. The liver is also the second most common site for metastatic spread of cancer. To assist in the diagnosis of these liver lesions non-invasive advanced imaging techniques are desirable. Magnetic resonance (MR) is commonly used to identify anatomical lesions, but it is a very versatile technique and also can provide specific information on tumor pathophysiology and metabolism, in particular with the application of MR spectroscopy (MRS). This may include data on the type, grade and stage of tumors, and thus assist in further management of the disease. The purpose of this review is to summarize and discuss the available literature on proton, phosphorus and carbon-13-MRS as performed on primary liver tumors and metastases, with human applications as the main perspective. Upcoming MRS approaches with potential applications to liver tumors are also included. Since knowledge of some technical background is indispensable to understand the results, a basic introduction of MRS and some technical issues of MRS as applied to tumors and metastases in the liver are described as well. *In vivo* MR spectroscopy of tumors in a metabolically active organ such as the liver has been demonstrated to provide important information on tumor metabolism, but it also is challenging as compared to applications on some other tissues, in particular in humans, mostly because of its abdominal location where movement may be a disturbing factor.

Key words: Metastasis; Metabolism; Proton magnetic resonance spectroscopy; Phosphorus magnetic resonance spectroscopy; Carbon-13 magnetic resonance spectroscopy; Magnetic resonance spectroscopy; Magnetic resonance

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INTRODUCTION

Primary liver cancer is the fifth most common malignancy in men and the eighth in women worldwide. In 2000, it was estimated that there were about 564 000 new cases of liver cancer worldwide, and a similar number of patients died as a result of this disease[1]. Also, the liver is

the second most common site for metastatic spread of cancer[2]. In fact, liver lesions are more likely to represent a metastatic tumor than a primary liver tumor[3,4]. For further clinical management a proper diagnosis is crucial. Currently tissue sample analysis by histopathology is the golden standard for the diagnosis of suspected cancer in the liver. However, taking biopsies for histopathological analysis has some disadvantages. Besides patient discomfort, there is a change that the needle misses the cancer foci. Also, the needle might loosen cancerous cells which could result in tumor dissemination outside the liver along the needle track[5].

Thus, non-invasive advanced imaging techniques are desirable to assist in the diagnosis of liver lesions. A major modality to obtain anatomical information is magnetic resonance (MR). The MR technique is very versatile and it also offers many possibilities to acquire more functional information. Among these, MR spectroscopy (MRS) is particular interesting as it can provide specific information on tumor pathophysiology and metabolism. This may include data on the type, grade and stage of the tumor, which thus can assist in further management of the disease.

In this review, we will summarize and discuss the available literature on MRS of primary liver tumors and metastases. We will focus on the main nuclei employed in MRS, proton (^{1}H), phosphorus-31 (^{31}P) and carbon-13 (^{13}C). Since knowledge of some technical background is indispensable to appreciate the impact of the results described in this paper we first give an introduction into the basics of MRS and some technical issues of MRS as applied to tumors and metastases in the liver.

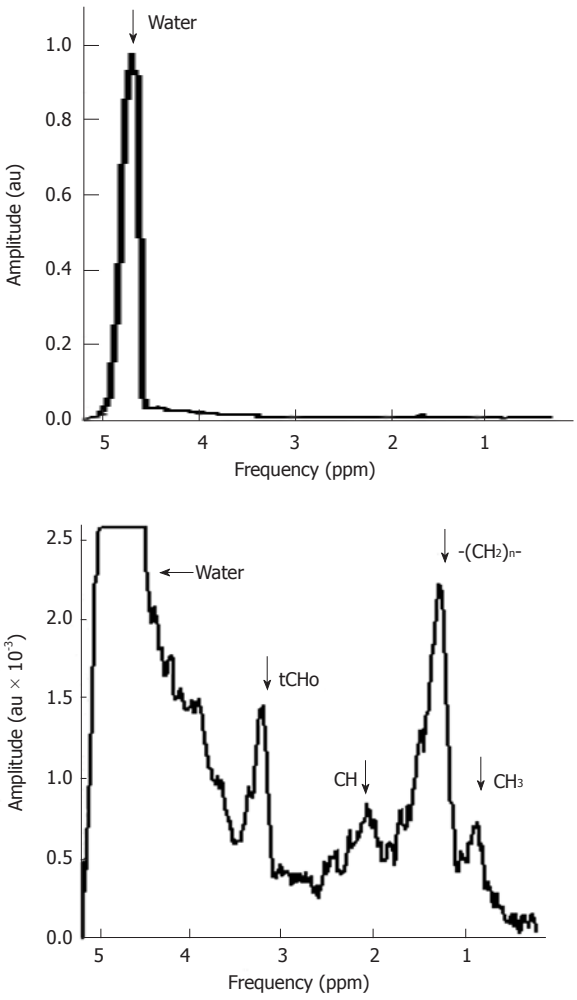

Figure 1 *In vivo* ^{1}H magnetic resonance spectra of human liver tissue obtained from a healthy volunteer on a 3.0T magnetic resonance system. Above: Spectrum with unsuppressed water signal; Below: Spectrum with partial suppressed water signal, showing the overlapping resonances for N-(CH$_3$)$_3$ protons at about 3.2 ppm occurring in choline compounds (tCho) and resonances for specific protons in lipids.

BASIC CONCEPTS OF MAGNETIC RESONANCE SPECTROSCOPY

In vivo MRS allows for the noninvasive measurement of the levels of some compounds in body tissues. It exploits the magnetic properties of certain atomic nuclei that are present in these molecules. The nuclei that are best accessible for *in vivo* MRS experiments are those of proton (^{1}H), phosphorus (^{31}P) and carbon-13 (^{13}C) atoms.

The following sections present a short introduction of MR spectroscopy to provide the reader with sufficient background to understand the biological and clinical applications of MR spectroscopy. For more in-depth information the reader is referred to other publications, see for example[6].

MR spectra

The key feature of MR spectroscopy is that certain biochemical compounds, mostly metabolites, can be identified in an MR spectrum by their specific spectral pattern, which is composed of one or more distinct signals. The intensity of the signal is proportional to the tissue amount of a certain nuclei and thus reflects the tissue levels of the compound in which it is present. An example of a typical *in vivo* ^{1}H MR spectrum of a healthy liver is shown in Figure 1. The horizontal axis of a spectrum represents the resonance frequency or chemical shift (both terms are explained below), the vertical axis represents the signal intensity. This spectrum is dominated by three main signals and in addition there are some smaller peaks, and broad underlying resonances. In the next few sections we explain in more detail how this spectrum is obtained and what particular information can be extracted from it.

Larmor frequency

Atomic nuclei with unpaired neutrons and/or protons, are detectable by nuclear magnetic resonance (NMR). As the nucleus is spinning around its axes and bears an electric charge it is associated with a magnetic dipole that can be seen as a tiny bar magnet (Figure 2). Outside a magnetic field these nuclear spins or tiny magnets have a random orientation; however, when placed in a strong constant external magnetic field B_0 they will become

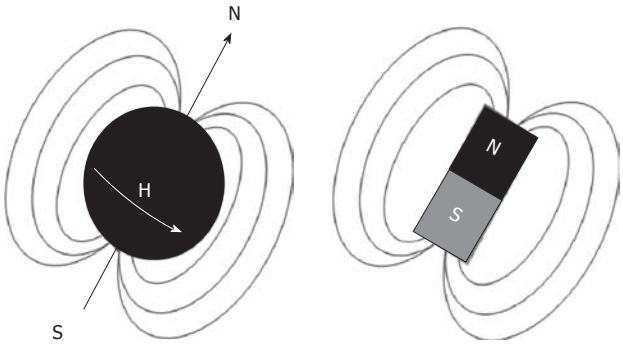

Figure 2 A ^{1}H nucleus spinning around its axes (left) can be regarded as a tiny bar magnet (right).

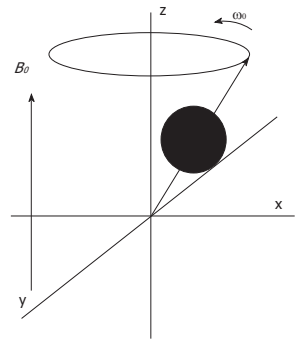

Figure 4 A spin precessing with the Larmor frequency around the axis of B_0 at a small angle with respect to this axis.

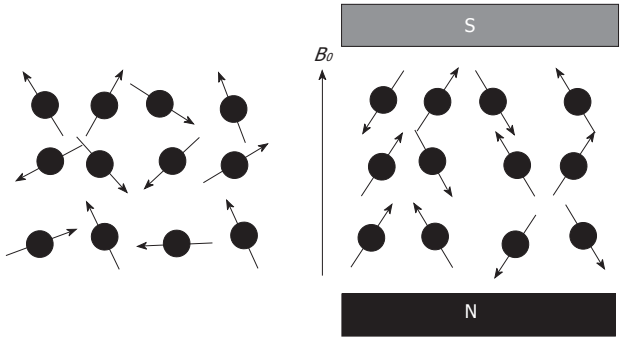

Figure 3 Outside a magnetic field spins have a random orientation (left) but spins inside a strong constant magnetic field B_0 will become aligned (right).

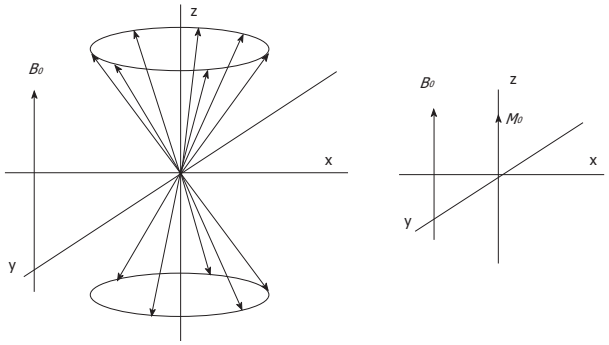

Figure 5 The direction of the main magnetic field B_0, is commonly placed along the z-axis and the magnetization along this axis is M_z, which at equilibrium, equals M_0. Left: Multiple individual spins precessing with the Larmor frequency around the axis of B_0. The spin vectors have a random, incoherent phase with respect to each other. Right: Magnetization vector M_z, which at equilibrium, equals M_0 (right).

aligned (Figure 3). The nuclear spins of the atoms ^{1}H, ^{31}P and ^{13}C can be oriented parallel or anti-parallel to B_0. However, the spins do not exactly align but are at an angle to B_0. This causes them to precess around the axis of B_0 with the so-called Larmor frequency $\nu_0 = \omega_0/2\pi = \gamma B_0/2\pi$ where the gyromagnetic ratio γ has a specific value for each nucleus (Figure 4). This implies that every type of nucleus has a different precession frequency, proportional to the B_0 field strength. At a field strength of 3T, which is commonly used for human applications, these frequencies for ^{1}H, ^{31}P and ^{13}C are: 127.7 MHz, 51.8 MHz and 32.1 MHz respectively, which is in the radiofrequency range.

Energy levels

The parallel and anti-parallel orientations are associated with a low and a high energy state respectively. The energy difference between the two spin states equals

$$E = h\,\gamma\,B_0 \qquad \text{[Equation 1]},$$

where h is Planck's constant ($h = 6.626 \times 10^{-34}$ J/s). At room temperature, there are slightly more spins in the lower energy level, N^{α}, than in the upper level, N^{β}. The distribution over these energy levels is given by Boltzmann statistics

$$N^{\beta}/N^{\alpha} = e^{-E/kT} \qquad \text{[Equation 2]},$$

where k is Boltzmann's constant (1.3805×10^{-23} J/K) and T is the temperature in Kelvin. The population difference results in a net macroscopic magnetization M_0. This so-called longitudinal magnetization is aligned parallel to B_0 (Figure 5). Only the net magnetization is detectable and its extent determines the achievable signal-to-noise ratio (SNR). At 1.5T and 37 ℃ (310 K), the population difference represents only a small fraction (about 10^{-6}) of the total spin population, which explains why MR is a relatively insensitive technique. It follows from Equations 1 and 2 that sensitivity can be improved with a higher magnetic field B_0 or a lower temperature.

Flip angle, free-induction decay, T_1 and T_2

The direction of the main magnetic field B_0, is commonly placed along the z-axis and the magnetization along this axis is M_z, which at equilibrium, equals M_0 (Figure 5). The longitudinal magnetization however is not detectable as it is "overruled" by the main magnetic field B_0. To detect the net macroscopic magnetization M_0, an radio frequency (RF) pulse with a magnetic field perpendicular to the main magnetic field B_0 and with a frequency equal to the precession frequency is sent with an RF transmitter coil. As a consequence of the applied RF pulse M_0 magnetization will rotate away from the z-axis toward the transverse plane (x-y plane). The angle to which the net magnetization is rotated relative to the main magnetic field direction is called the flip angle. A

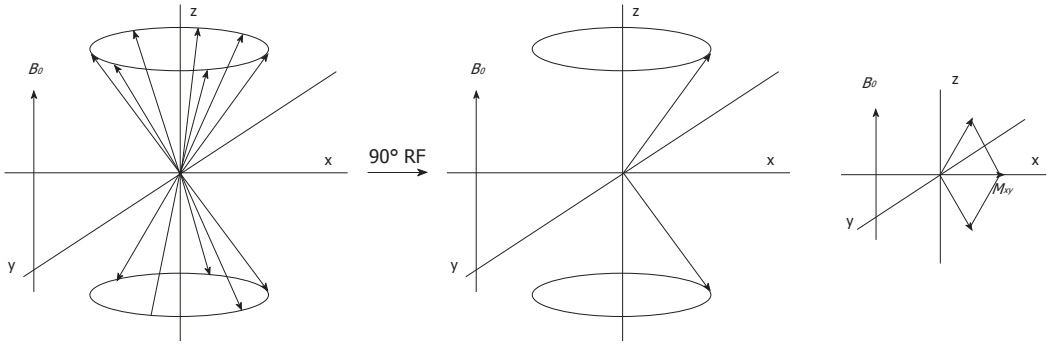

Figure 6 Multiple spins precessing with the Larmor frequency around the direction of B_0 (left). After a 90° radio frequency (RF) pulse, the spin population of the energy levels become equal and the spins precess coherently (middle). The magnetization vector M_0, rotates into the transverse (x-y) plane (right).

so called 90° RF excitation pulse will therefore rotate M_0 into the transverse plane. The spin population of the energy levels becomes equal and spins precess coherently (Figure 6). However, after the RF excitation pulse the spins start to return towards the original energy level distribution and towards incoherent precession (dephasing) and after a while the system will be in equilibrium again. The time constant which describes how the magnetization returns to the original longitudinal alignment is called the spin lattice relaxation time T_1: $M_z = M_0 (1\text{-}e^{-t/T1})$. The time constant which describes the return to incoherent precession is called the spin-spin relaxation time T_2: $M_{xy} = M_{xy0}\, e^{-t/T2}$. By definition T_1 is longer than T_2.

After the RF pulse, the transverse component of the M_0 magnetization precesses with the Larmor frequency at resonance and will induce a current in the RF coil which is now switched to receive mode. The decaying signal of this component is called the free-induction decay (FID) response signal. This digitally recorded FID signal is mathematically converted by a Fourier transform from the time to the frequency domain, which results in a so called MR spectrum, that may contain one or more resonances, signals or peaks at particular frequencies.

Shielding and chemical shift

An MR spectrum of the liver would not be very interesting if all nuclei of a certain type resonate at the same frequency. Fortunately, each nucleus in a given molecule is shielded from the main field by a weak opposing field from the surrounding electrons, induced by and also proportional to B_0. The amount of shielding by these electrons highly depends on the chemical environment of the nucleus. This shielding, which is generally unique for each chemically distinguishable site in a molecule, is expressed as the shielding constant σ, and the total effective field experienced by a given nucleus is: $B_{eff} = B_0 (1\text{-}\sigma)$. The resulting change in resonance frequency $\nu_{eff} = \omega_{eff}/2\pi = \gamma B_0 (1\text{-}\sigma)/2\pi$ relative to that of a chosen reference compound, ν_{ref}, is generally referred to as the chemical shift $\sigma = (\nu_{eff} - \nu_{ref})/\nu_{ref}$ expressed in units of ppm (1 ppm = 100 Hz at ν_0 = 100 MHz). Thus the chemical shift is the key property of MR spectroscopy which enables detection of a wide variety of chemical groups and metabolites containing these groups. Special RF pulses are used to excite a band of frequencies covering the chemical shift range of a particular nucleus in a biological sample.

^{1}H MR SPECTRUM OF THE LIVER EXPLAINED

As shown in Figure 1 the ^{1}H spectrum of the liver is dominated by 3 peaks. The peak on the right originates from the ^{1}H nuclei in methylene groups (-CH_2-) in lipids, the peak in the middle originates from the ^{1}H nuclei in the three methyl groups (CH_3) of choline containing compounds [$(CH_3)_3N^+CH_2CH_2O$-], and the peak on the left originates from the ^{1}H nuclei in water (H_2O). The electronegative oxygen atom in the water molecule shifts the electron density away from the ^{1}H nuclei, leading to a reduced shielding and thus to a higher resonance frequency compared to ^{1}H nuclei in the methylene groups of lipids. Thus ^{1}H nuclei in water have a higher chemical shift value then the ^{1}H nuclei in the methylene groups from lipids (compared to ^{1}H nuclei in a reference compound at 0 ppm) and appear on the left side. Note that the chemical shift scale on the horizontal axis increases from right to left.

The peak area rather than its amplitude is proportional to the amount of ^{1}H nuclei in the same chemical environment and thus the tissue content of that chemical group or metabolite. Quantification of metabolite concentrations is performed by comparing peak areas with those of known substances and concentrations.

The content of water in the liver is much higher than that of metabolites. Hence, the proton signals of the latter have a much lower SNR (Figure 1). For this reason signal averaging is usually required and it is also needed to measure larger voxels than commonly done with MR imaging of water.

WATER SIGNAL SUPPRESSION

As the water resonance in ^{1}H MR spectra is at least 200 times more intense than the resonances of hydrogen

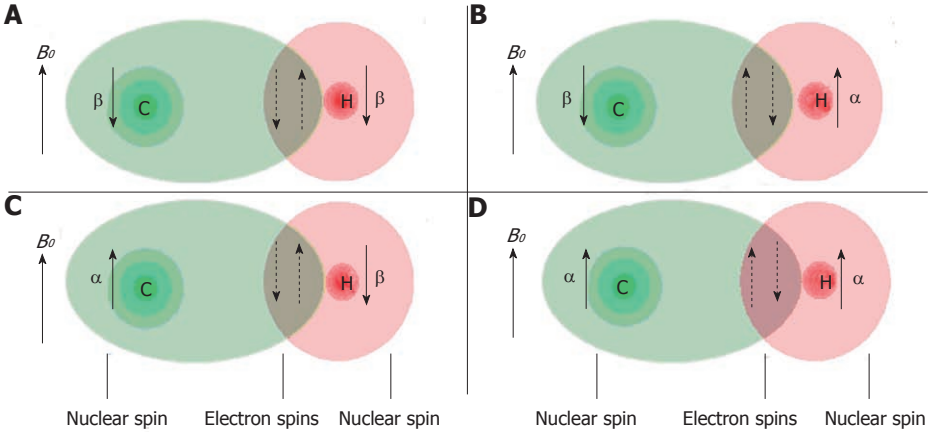

Figure 7 Two bonded nuclei, carbon (C) and hydrogen (H) in decreasing energy levels associated with spin states: ββ (A), βα (B), αβ (C) and αα (D). The electron clouds around each nucleus are indicated in light green and red. Arrows indicate individual spin states.

containing metabolites (Figure 1) it may hamper the proper detection of metabolite signals, e.g., by artifacts such as side bands of this huge signal[7], the suppression of the water signal is commonly performed. There are many ways to do this: a well-known sequence is chemical shift selective water suppression[8]. A frequency selective RF pulse excites the water spin magnetization into the transverse plane after which all coherences are dephased by magnetic field gradients. The spectrum shown in Figure 1 was obtained with only partial water signal suppression.

MAGNETIC FIELD HOMOGENEITY

As the purpose of MR spectroscopy is to separate signals with different chemical shift it is crucial for its proper application that good magnetic field homogeneity is obtained over the object of interest. However, due to the many different tissue types and air containing compartments, the magnetic field in the human body is usually not very homogeneous, leading to broadened spectral lines, which may overlap. Therefore optimizing field homogeneity (a process called shimming) is usually a required step in an MR spectroscopy experiment. Good homogeneity is most important in ^{1}H MRS as it has a relatively small chemical shift range and needs a well separated and defined water signal for proper suppression and to avoid artifacts. By selecting small volumes of interest, a limited amount of different tissues are included which will result in a more homogeneous magnetic field. The requirements for homogeneity are much less strict for ^{31}P and ^{13}C MRS because resonances in their spectra are more separated (larger spectral dispersion).

SPIN-SPIN COUPLING AND DECOUPLING

Nuclei which are close to one another exert an influence on each other's effective magnetic field through electrons in chemical bonds (Figure 7). If the distance between non-equivalent nuclei is less than or equal to three bond lengths, this effect may be observable in the *in vivo* MR spectrum. Instead of one peak for a certain nucleus several smaller peaks are observed in the spectrum. This interaction of nuclei is often referred to as scalar coupling, J coupling or spin-spin coupling. Because of this splitting the signal to noise decreases and spectral interpretation may become more complicated. However, the specific pattern may also be helpful to identify specific molecular groups and with special pulse sequences the phenomenon of spin-spin coupling may be used to identify the resonances of these groups in the presence of other overlapping resonances (so-called editing).

Spin-spin couplings are expressed in Hz. A typical value for a three bond proton-proton coupling in metabolites is 7 Hz. For example this can be used to identify the methyl doublet resonance of lactate in ^{1}H MR spectra. At magnetic fields above about 2T, line width broadening will obscure the direct visualization of this coupling. Two bond ^{31}P - ^{31}P couplings occur at about 17 Hz in adenosine triphosphate (ATP). Heteronuclear couplings commonly dealt with in *in vivo* MRS are between protons and ^{31}P or ^{13}C. To improve signal to noise and spectral resolution the spin-spin splitting can be removed by a technique called decoupling. Special RF pulses are used to irradiate selected resonance(s) of nuclear spins such that their field directionality is averaged out. As a result nearby spins in a molecule experience no field of these irradiated spins anymore and the resonance splitting disappears.

Decoupling is important in MRS of ^{31}P and ^{13}C as these atoms often show split resonances due to nearby hydrogen atoms. Irradiation of these protons for decoupling increases the signal to noise ratio and improves spectral resolution. Irradiation also induces a through space effect called nuclear overhauser enhancement (NOE), which can increase signal intensity even more[9-11]. Typical enhancement values reached *in vivo* by NOE are 1.3-2.9 and 1.4-1.8 for ^{13}C-^{1}H and ^{31}P-^{1}H interactions respectively[6].

DYNAMIC NUCLEAR POLARIZATION

^{13}C labeled (enriched) substrates combined with ^{13}C MRS have traditionally been used to monitor metabolic conversions during steady-state conditions and even has been used to assess active metabolism in human tumors[12-14]. The relative low sensitivity of ^{13}C MRS prevented imaging of these processes. However, with polarization transfer techniques sensitivity can be enhanced[14-16].

Recently a new method has been introduced in biomedical MR, dynamic nuclear polarization (DNP), that mostly makes use of ^{13}C MRS, to image metabolic conversions at reduced acquisition times (in an order of minutes or less). Inspection of Equation 2 (above) reveals that decreasing the temperature also will increase the population differences between energy levels. DNP is a hyperpolarization technique that increases the spin polarization obtained at room or body temperature in traditional MR, by orders of magnitude by cooling the sample to very low temperatures and using selective microwave irradiation for efficient transfer of spin polarization from electron spin to nuclear spin[6,17,18]. The substrate is then rapidly transferred to body temperature for administration. The major disadvantage of DNP is that the polarization decay is determined by the spin-lattice relaxation time T_1 of the nucleus (20-40 s for a ^{13}C nucleus in a carboxyl group). The consequence is that only conversions in which substrates are rapidly taken up by tissues and metabolized within minutes can be imaged successful. With efficient uptake usually one or two metabolic steps can be imaged, before the signal has decayed away.

LOCALISATION

To analyze different regions of interest in the liver with healthy tissue or tumor lesions by *in vivo* MRS it is required that only signals that originate from these locations appear in the spectra. This can be achieved in different ways. The most rudimentary one is to use only a surface coil on the body adjacent to the liver with a simple pulse sequence globally selecting the area next to the coil. However, in most cases a better localization is desired. More advanced spatial localization is possible with single voxel or multi-voxel methods.

Single voxel localization

The most common single voxel localization techniques are Image Selected *In Vivo* Spectroscopy (ISIS)[19], Stimulated Echo Acquisition Mode (STEAM)[20] and Point Resolved Spectroscopy (PRESS)[21].

ISIS uses three frequency-selective inversion pulses, in the presence of three orthogonal magnetic field gradients. By turning on and off the inversion pulses, according to an encoding scheme, eight different scans are recorded. Adding and subtracting the different scans will add signal from the desired location while canceling signal from other locations. A disadvantage of ISIS is its sensitivity to motion as eight scans need to be obtained for a full 3D localization. ISIS is rarely used for ^{1}H MRS, because of potential artifacts such as those arising from incomplete water signal suppression. However, it is the favored method in ^{31}P MRS localization as effects due to relatively rapid T_2 decay and to J-coupling are avoided.

For ^{1}H MRS the most common single voxel localization techniques are STEAM and PRESS, which both use three spatially slice selective RF pulses to produce an echo signal from a well-defined region. STEAM uses 90°-90°-90° pulses and PRESS 90°-180°-180° pulses to define three orthogonal slices. Only signal from the volume of interest remains in the final echo. In STEAM 50% of the original signal is lost as the second 90° pulse only rotates half of the transverse magnetization to the longitudinal axis, while the other half is dephased by crushers. The PRESS technique retains full signal intensity, but the minimal possible TE is commonly larger than for STEAM.

Multi voxel localization

Multi voxel localization allows the detection of localized spectra from a multidimensional array of locations. Disadvantages compared to single voxel localization concerns some more magnetic field inhomogeneities due to the many different tissue types in the field of view, intervoxel contamination, and the minimally required number of scans that may end up in long acquisition times. Spectroscopic imaging techniques acquire the signal from multiple voxels by using phase encoding gradients[22], analogous to the phase encoding technique used in MR imaging. The nominal voxel size is the field of view divided by the number of phase encoding gradient steps. The actual voxel size can deviate substantially from the nominal value as the signal is sampled only over a finite time. This introduces intervoxel contamination due to the characteristics of the Fourier transform. This voxel bleeding can be decreased by apodization functions like those with Gaussian or Hamming shapes, however at the expense of decreased spatial resolution.

Conventional encoding of $N_1 \times N_2 \times N_3$ volume elements (voxels) requires $N_1 \times N_2 \times N_3$ acquisitions. A typical $16 \times 16 \times 16$ dataset obtained with a repetition time of 2000 ms and 4 averages would require a measuring time of $(16 \times 16 \times 16 \times 2000 \times 4/3600 = 9$ h). Therefore techniques are developed that increase the temporal resolution, e.g., by special k-space trajectories. For example "circular 2D or spherical 3D k-space sampling with k-space apodization during acquisition". This also reduces the total acquisition time by spending less time acquiring the high k-space coordinates and more time acquiring the low k-space coordinates. Other methods are based on fast magnetic resonance imaging (MRI) sequences, e.g., EPI, RARE, spiral and steady-state sequences[6].

QUANTIFICATION

Although the tissue level of metabolites is proportional

to the area under its signal curve in the spectrum, it commonly requires some corrections and calibration with a signal of known concentration to obtain an absolute number (e.g., in mmol/L).

Such a reference signal maybe that of water or of another metabolite in the liver assuming a stable and known value for its tissue level. In the liver, the unsuppressed water signal is often used as an internal reference after correction for T_1 and T_2 relaxation. However, dietary regimes and liver pathologies may affect the amount of water. Li *et al*[23] reported a 1.8 fold difference in five normal liver studies between the largest and smallest water signal intensity obtained from localized liver tissues. In four hepatocellular carcinoma studies, they observed a 3.2-fold difference between the largest and smallest water signal intensities obtained from the localized liver tumors. Lipid peaks exhibited even larger variations than did the water peaks.

External phantoms with known concentrations sometimes are also used for calibration purposes, but this may be not so practical in a clinical environment. In addition differences in coil loading have to be taken into account in this approach. To avoid correction and calibration issues spectral quantities are sometimes also assessed as ratio's between integrals of signals of different compounds.

MOTION AND OTHER ARTEFACTS

Motion can lead to voxel misregistration and outervoxel contamination. Motion of tissue through inhomogeneous fields (e.g., air in the lungs) results in broadening of the spectral resonances. Broadening of the resonances increases the risk of signal overlap and also lowers the signal to noise ratio. Compared to other organs like brain and skeletal muscle, MR spectroscopy of the liver is challenging as there are potential field inhomogeneities and artifacts caused by respiratory movement, cardiac and aortic pulsations[24-26]. Although often applied, breath-hold acquisitions may be problematic. Long acquisition times are needed to increase the SNR and, since a breath-hold period can only last for about 15 s in patients, the acquisition will require multiple breath-hold periods. Even when the acquisition is performed at end-expiration, there is no guarantee that the tissue is at exactly the same position, leading to outervoxel contamination. Respiratory and cardiac gating can be applied to reduce motion artifacts at the cost of an increased scan time. Another option is to align individual spectra and/or exclude bad spectra before averaging during post processing. Some liver pathologies, e.g., due to long-term total parenteral nutrition may induce iron accumulation in the liver, which will result in field inhomogeneities and broadening of the spectral resonances.

REPRODUCIBILITY

In order to predict treatment outcome or monitor therapy, differences in *in vivo* MR spectroscopy outcome parameters should reflect true differences in tumor biology and not differences induced by variations in the MRS protocol or interfering body physiology. This issue is relevant, since the time of MR scanning during the day (e.g., before or after a meal) or differences in eating patterns might already influence the metabolic activity and thus the concentrations of metabolites in the liver. Large inter- and intrapatient variability of MRS outcome parameters have been described[23].

^{1}H MR SPECTROSCOPY

In MR, hydrogen (proton) is the most commonly studied nucleus. Compared to other MR sensitive nuclei it has the highest sensitivity and occurs at 100% isotopic abundance. Almost all metabolites in the human body contain protons. Therefore, in principle a large amount of metabolites can be investigated. However, in practice, sensitivity restrictions set the *in vivo* detection limit of metabolites to a minimum tissue concentration of about 0.1 mmol/L. An advantage of ^{1}H MRS is that it uses the same nucleus as MRI techniques. Therefore, it can be performed with the same hardware and there is no need for special equipment. The major drawback of ^{1}H MRS is the relatively small chemical shift range (about 10 ppm) for the many resonances of *in vivo* detectable compounds, resulting in limited spectral resolution. Moreover, these have to be resolved from a dominating water peak, in certain cases a large lipid peak, and at short echo times a high baseline due to macro-molecules.

Metabolites visible in ^{1}H MR spectra of liver tumors

Lipids: Outside the brain ^{1}H MR spectra of tissues usually show large signals of mobile lipids, mostly triglycerides: in particular a methylene peak at 1.2 ppm with smaller methylene peaks between 2.1 and 2.4 ppm and a peak for methyl protons at 0.9 ppm (Figure 1). Triglycerides occur in fatty liver, but also may be a marker of membrane breakdown and can be seen in tumors, abscesses and other pathological processes[27].

Lactate: Due to the Warburg effect tumor cells obtain relative less energy than normal cells from oxidative phosphorylation and have a more glycolytic character[28,29]. Pyruvate, the end product of glycolysis, is converted into lactate, which is further promoted by hypoxic conditions. Therefore MR spectra of tumor tissue often show signals for lactate. The three equivalent methyl protons of lactate give rise to a resonance at 1.31 ppm, which is a doublet due to coupling with the methylene proton, while the single methylene proton resonates as a quartet at 4.10 ppm due to coupling with the methyl protons. In liver and tumor tissue, the lactate signal at 1.3 ppm will overlap with large lipid resonances. However, with so-called spectral editing techniques it is possible to separate the lactate signal from the lipid signals.

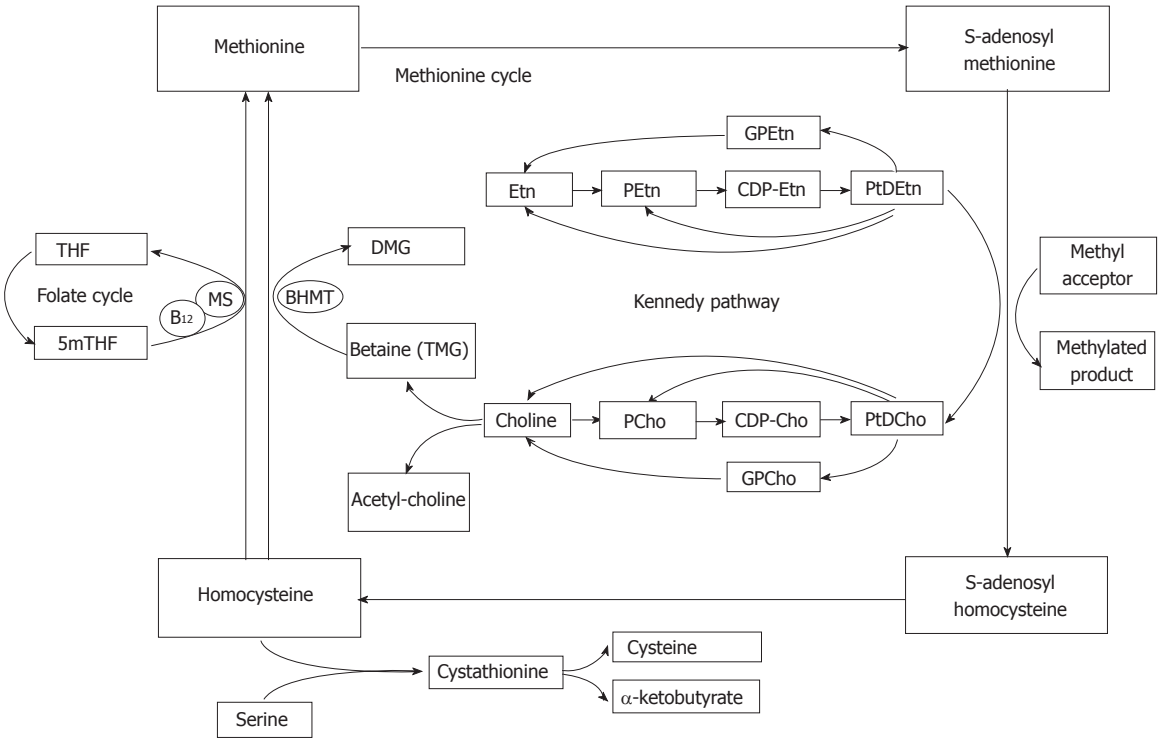

Figure 8 Simplified schematic overview of choline and ethanolamine metabolism including parts of the Kennedy pathway, the methionine and folate cycle. B12: Vitamin B12; BHMT: Betaine-homocysteine methyltransferase; CDP-Cho: Cytidine diphosphate-choline; CDP-Etn: Cytidine diphosphate-ethanolamine; DMG: Dimethylglycine; Etn: Ethanolamine; GPCho: Glycerol-3-phosphorylcholine; GPEtn: Glycerol-3-phosphorylethanolamine; MS: Methionine synthase; PCho: Phosphorylcholine; PEtn: Phosphorylethanolamine; PtdCho: Phosphatidylcholine; PtdEtn: Phosphatidylethanolamine; THF: Tetrahydrofolate; 5mTHF: 5-methyltetrahydrofolate; TMG: Trimethylglycine (betaine).

Creatine: Creatine (Cr) and phosphorylated creatine (PCr) play an important role in energy metabolism of many tissues. PCr serves as a spatio-temporal energy buffer, maintaining a constant level of ATP, facilitated by the creatine kinase reaction. The methyl protons of Cr and PCr resonate at about 3.03 ppm and the methylene protons resonate at approximately 3.9 ppm. Under normal conditions, the concentration of total creatine is relatively constant in muscle and brain and therefore often used as an internal reference. However, decreased Cr levels have been observed in tumors and other pathologies. Furthermore, hepatocytes do not express creatine kinase under normal circumstances[30], and therefore no creatine peak will be visible in the spectrum of healthy liver tissue. *In vitro* experiments at 9.4T have shown 5-10 times increased levels of Cr in liver metastasis compared to normal liver tissue[31].

Choline and ethanolamine containing compounds

The signals of choline containing compounds in MR spectra have been used as key biomarkers to identify malignant tumors[32-34]. *In vivo* ^{1}H MR spectra of the liver show the N-trimethyl [$^{+}N(CH_3)_3$] resonances of choline compounds at about 3.2 ppm. This resonance is also known as the total choline (tCho) peak as it may originate from several different choline compounds. The relative increase in the tCho signal seen in human tumors is due to an abnormal choline uptake and/or metabolism related to cell membrane turnover. However, metabolism of choline containing compounds in tissue cells is complex. Although far less prominent in ^{1}H MR spectra than choline, ethanolamine signals may also contribute to a characteristic spectral profile of tumor tissue. Therefore some important biochemical pathways involving choline metabolism and the closely related metabolism of ethanolamine in the liver are briefly described (Figure 8).

Choline and ethanolamine metabolism

Choline is a key precursor molecule in several metabolic pathways. It can be acetylated, oxidized, phosphorylated or hydrolyzed. Choline oxidation plays a major role in the provision of methyl groups *via* its metabolite, trimethylglycine (betaine) that participates in the synthesis of S-adenosylmethionine (SAM). Methylation reactions are involved in the biosynthesis of lipids, the regulation of several metabolic pathways, and detoxification in the body. Choline phosphorylation results in compounds such as phosphatidylcholine (PtdCho), lysophosphatidylcholine, choline plasmalogen, and sphingomyelin which are essential for structural integrity and signaling in cell membranes[35-38].

PtdCho, the major phospholipid component of cells is derived from the Kennedy pathway[39], which has two branches, one *via* cytidine 5'diphospho (CDP)-choline and the other *via* CDP-ethanolamine (Figure 8). In the CDP-choline branch choline is initially converted

to phosphorylcholine (PCho) and after some steps to PtdCho which can be converted into choline or into PCho again[36]. Alternatively, phosphatidylethanolamine (PtdEtn) is generated *via* the CDP-ethanolamine branch, employing similar biochemical reaction steps. The resulting PtdEtn can be methylated, using SAM as the methyl donor, to PtdCho[36-38,40]. The methylation pathway is, however, only relevant in liver. In rat hepatocytes it accounts for 20%-40% of PtdCho synthesis[41]. Besides entering the CDP-choline branch of the Kennedy pathway, choline can also enter another major pathway in the liver in which it is oxidized into betaine[42-44].

Contributions to the tCho peak

The contribution of the nine methyl protons of free choline, which resonate at 3.19 ppm, to the tCho peak is limited as the concentration of free choline is usually low. Another potential contribution to the tCho peak may come from PtdCho, which makes up a very high proportion of the cell plasma membrane. However, it is a large molecule with a relatively short T_2 relaxation time that becomes even shorter by being incorporated into a membrane. Therefore, it is almost invisible in the *in vivo* ^{1}H MR spectrum. Nevertheless, some evidence suggests that PtdCho may contribute to the tCho signal[45]. Precursors of PtdCho such as PCho and phosphorylethanolamine (PEtn) are more likely to contribute to ^{1}H MR spectra as these are small molecules with long T_2 relaxation times. Experimental evidence suggests that the tissue levels of PCho and PEtn increase during cell proliferation and tCho levels also have been correlated with tumor aggressiveness[46]. In addition to PCho and PEtn [together called phosphomonoesters (PME)] their glycerol derivatives glycerol-3-phosphorylcholine (GPCho) and glycerol-3-phosphorylethanolamine (GPEtn) [together called phosphodiesters (PDE)] also contribute to the tCho signal. Besides protons of choline and ethanolamine containing compounds, protons from other metabolites might also resonate around 3.2 ppm, e.g., glucose at 3.23 ppm, myo-inositol at 3.27 ppm, and taurine at 3.25 ppm. In liver and kidney the resonance at about 3.26 ppm is almost entirely composed of proton signals of betaine (trimethylglycine)[6].

Liver tumors and metastases

***In vitro* high field ^{1}H MR spectra of the liver:** Soper *et al*[47] performed a diagnostic correlation between MR spectra and histopathology. They analyzed liver tissue specimens from 54 patients undergoing partial or total hepatectomy. The samples included 31 normal, 59 cirrhotic and 32 hepatocellular carcinoma (HCC) histologically confirmed tissues and were analyzed by ^{1}H MRS at 8.5 Tesla. They found reduced amounts of lipids and carbohydrate residues and increased tCho in HCC compared to all normal and all cirrhotic liver tissue. Cirrhotic liver tissue and HCC were distinguished with a sensitivity and specificity of 95.8% and 88.9%, respectively. Lactate signals of variable intensity were found at 1.3 ppm, probably resulting from anaerobic metabolism after excision.

***In vivo* liver ^{1}H MR spectrum:** *In vivo* ^{1}H MRS is characterized by a much poorer spectral resolution and SNR than *in vitro* ^{1}H MRS (see above, technical issues). Kuo *et al*[48] investigated the value of *in vivo* ^{1}H MRS in the assessment of large focal hepatic lesions. They included 43 consecutive patients and 8 normal volunteers in a prospective MRS study. MRS was performed at 3.0T with shallow and regular breathing. Single voxel PRESS with TE = 30 ms, TR = 1500 ms, 256 averages, was used to select a volume of 2 cm × 2 cm × 3 cm. The voxel of interest was located in the largest solid portion of hepatic tumors in patients. Healthy liver data was collected from an area at the centre of the right hepatic lobe for normal volunteers, or in an uninvolved area of the right hepatic lobe in patients. Patients with diffuse-type HCC, with focal nodular hyperplasia and obvious fatty infiltration, and histological unconfirmed lesions were excluded. Thirty-three lesions (21 HCC, 2 angiosarcomas, 1 lymphoma and 9 hemangiomas) were included. They found that malignant tumors had elevated tCho resonances compared to uninvolved liver or benign tumors, but the difference in mean tCho/lipid ratio between malignant tumors or uninvolved liver did not reach statistical significance. Several factors may have contributed to these results. First of all, the tumors in this study may have contained significant necrotic areas with less viable cells. This may have diluted more prominent changes observed in areas of rapid cell turnover, within viable tumor tissue and may have caused low signal to noise for metabolite signals leading to larger errors. Physiological motion due to breathing and cardiac movement will have contributed, especially if the tumor was located in the left lobe or at the extreme end of the right hepatic lobe. Finally, three different tumor types were included in the malignant group, which may have resulted in a variation of different metabolites in phospholipid metabolism, and thus in a more variable tCho resonance. Thus, *in vivo* ^{1}H MRS is technically feasible at 3.0T for the evaluation of focal hepatic lesions, but the clinical application of the measurement protocol used by Kuo *et al*[48] is limited as normal liver, benign and malignant tumors cannot be clearly differentiated.

In the second part of their study Kuo *et al*[48] attempted to measure metabolic changes in HCC after transcatheter arterial chemoembolization (TACE). Eight HCC were evaluated before and two to five days after TACE. The tCho peak at 3.2 ppm was significantly decreased while the lipid and water signals at 1.3 and 4.7 ppm respectively, were increased. The mean tCho/lipid ratio significantly decreased from 0.23 ± 0.11 before to 0.01 ± 0.00 after TACE treatment. One of the post-TACE lesions showed recurrence three months later and MRS also revealed an elevated tCho/lipid ratio at that stage. Therefore, ^{1}H MRS at 3.0T may be used for treatment monitoring.

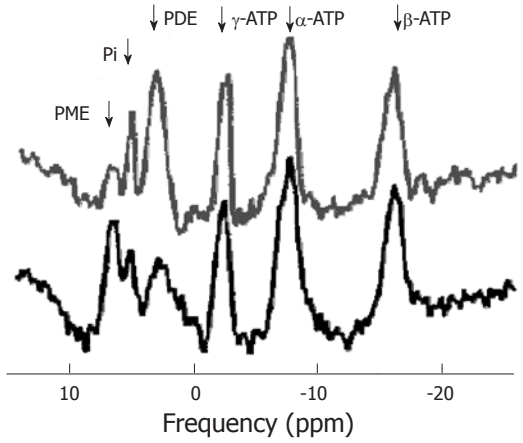

Figure 9 ***In vivo*** **^{31}P magnetic resonance spectra of human liver tissue obtained from a healthy volunteer (top) and from a patient with hepatocellular carcinoma (bottom).** PME: Phosphomonoesters; PDE: Phosphodiesters; Pi: Inorganic phosphate; ATP: Adenosine triphosphate. (Reproduced with permission of John Wiley and Sons, www.interscience.wiley.com, from[64].)

Fishbach *et al*[49] improved the MRS acquisition and processing protocol compared to previous studies by introducing a control of respiratory motion using breath-hold acquisitions and an abdominal compression belt. They also applied dedicated pre- and post-processing including automatic phase and frequency correction based on the residual and the unsuppressed water signal in order to remove potential distortions mainly introduced by motion. Apart from 39 volunteers, they included 55 patients with advanced cancer with lesions of more than 3 cm in diameter in their study (22 metastases of colorectal cancer, 11 hepatocellular carcinomas, 9 metastases of breast cancer, 3 metastases of pancreatic cancer and 1 metastasis of prostate cancer). Liver spectra were acquired at 3.0T using a body transmit/receive coil. Breath-hold at end-expiration spectra were acquired with the single voxel (2 cm × 2 cm × 2 cm) PRESS technique with TE=35 ms, TR=2000 ms, 128 averages and 16 additional unsuppressed water reference lines. The intra-individual reproducibility of this ^{1}H MRS acquisition in the liver was tested in 25 patients and volunteers and judged to be satisfactory. In total 186 spectra were acquired and 27 spectra had to be discarded because they did not meet the predefined quality specifications. The remaining 113 spectra were measured in normal-appearing parenchyma of 37 patients and 39 volunteers.

Although, remarkably, tCho signals relative to those of water seemed to be lower in metastatic lesions compared to normal liver tissue, no significant differences were observed between malignant liver tumors and normal liver parenchyma for any of the parameters analyzed, in particular tCho signals relative to water and lipid signals. This was attributed to the large variability of normal values.

The divergent results observed in the above mentioned studies might also be due to the multiple contributions from the unresolved tCho signal. In normal liver tissue low concentrations of PMEs and high concentrations of PDEs have been shown while in tumor tissue elevated levels of PMEs and decreased levels of PDEs have been observed[50]. This implies that in tumor tissue increased levels of PMEs may be canceled out by decreased PDEs, resulting in an unchanged overall tCho level. Different tumor types might also have divergent contributions to the unresolved tCho signal. In addition, alterations of the metabolite concentrations might not be due to malignancy, but due to proliferating healthy tissue such as a regenerating liver, benign tumors, and even some degenerative pathologies. With ^{31}P MRS PME and PDE signals can be studied separately as discussed below.

^{31}P MR SPECTROSCOPY

After ^{1}H MRS, phosphorus-31 MR spectroscopy is the most commonly used MRS technique to study tumors *in vivo*. Phosphorus has an MR sensitivity of 6.6% compared to proton. However, the chemical shift dispersion of its signals observed *in vivo* is larger (about 30 ppm *vs* about 10 ppm), resulting in a better spectral resolution. Also, ^{31}P MRS is capable of detecting some key metabolites in tissue energy metabolism such as ATP, PCr, and inorganic phosphate (Pi). In addition some important metabolites involved in membrane metabolism such as PCho and PEtn (together called PME) and their glycerol derivatives GPCho and GPE (together called PDE) may be resolved. In this respect ^{31}P spectra (Figure 9) are more informative than ^{1}H MR spectra in which signals of all choline compounds (tCho) usually are observed unresolved at about 3.2 ppm. Furthermore, from ^{31}P MR spectra physiological parameters like intracellular pH can be deduced from the chemical shift of the Pi resonance. The phosphoryl resonances from large and membrane bound compounds may only show up in a phosphorous-31 MR spectrum as broad underlying baseline signals due to their very short T_2 values[51].

Unfortunately, due to a lower sensitivity and less favorable spin relaxation the spatial resolution of ^{31}P MR spectra is an order of magnitude less than that of ^{1}H MRS. Higher magnetic fields provide improved experimental conditions for ^{31}P MRS.

Metabolites visible in ^{31}P MR spectra of liver tumors

Phosphocreatine: The largest peak in ^{31}P MR spectra of muscle, brain and other tissues originates from PCr. Its spectral position is used as an internal chemical shift reference and commonly has been assigned a chemical shift of 0.00 ppm. PCr is, however, not detectable in spectra of healthy liver since hepatocytes do not express creatine kinase under normal circumstances[30]. Tumors, however, might express creatine kinase and show some PCr.

Adenosine triphosphate: ATP is the main direct energy supply within cells. ATP consists of adenosine and three phosphate groups. The phosphoryl groups, starting with those closest to the adenosine moiety, are referred to as α, β,

and γ phosphates. ATP is produced by ATP synthase from inorganic phosphate and adenosine diphosphate (ADP) or adenosine monophosphate (AMP). Multiple processes in the cell can split ATP into ADP or AMP and inorganic phosphate, and use the energy that is released. At a pH of 7.2, with full magnesium complexation, the resonances of ATP appear at -7.52 ppm (α), -16.26 ppm (β), and -2.48 ppm (γ). The ATP resonances may overlap with the signals of other nucleotides: uridine triphosphate (UTP), guanosine triphosphate (GTP) and cytidine triphosphate (CTP). Therefore these resonances are sometimes referred to as nucleoside triphosphate (NTP), although the others usually occur at much lower concentrations.

Inorganic phosphate: Like the level of ATP that of inorganic phosphate (Pi) reflects the cellular phosphorylation potential. The chemical shift of Pi and some other phosphorus containing compounds is dependent on the intracellular pH (pHi) and magnesium concentrations[52]. The protonation or complexation with magnesium of phosphate affects the chemical environment of the ^{31}P nucleus and hence its chemical shift. As proton exchange is fast on the NMR timescale, the resonance frequency is indicative of the relative amount of protonated and unprotonated molecules, and hence the pH can be deduced. The shift in resonance of Pi relative to PCr is most commonly used as it has a large dependence in the physiological pH range whereas the chemical shift of PCr is constant in this range. At a pH of 7.2 and normal magnesium level it occurs at 5.02 ppm. The accuracy of pH determination from the Pi-PCr shift is typically 0.05 pH units[6]. However, as PCr is not detectable in the normal liver the α-ATP resonance is used instead as a reference.

Tumor pH: Due to the Warburg effect and/or hypoxic conditions tumor cells preferentially convert glucose to lactic acid. Lactic acid is largely dissociated *in vivo* to H^+ and lactate$^-$. Normal, as well as tumor cells, have multiple systems to continuously export H^+ ions to maintain a constant pHi, as well as a system for exporting lactic acid (but not lactate). This results in a neutral, or a slightly alkaline pHi of intact (tumor) cells. However, since tumors may be poorly vascularized the extracellular tumor pH (pHe) of tumors is more commonly acidic[53-59].

Phosphodiesters: Signals of PDE occur around 3 ppm in ^{31}P MR spectra. Tumor tissue sometimes contains significantly lower concentrations of PDE than healthy liver tissue. Some *in vitro* studies show that the PDE levels increase with decreasing growth fraction of the tumor. This suggest that PDE signals may be dominated by breakdown products of phospholipids[60], and the concentration may be an indicator of the necrotic fraction in tumors associated with phospholipid catabolism[61]. The phospholipid derivatives, particularly GPCho and GPEtn, were found to contribute to the PDE resonance[61]. Their phosphor spins resonate at 2.76 ppm and 3.20 ppm respectively.

Phosphomonoesters: Resonances of PME occur at about 6 ppm in ^{31}P MR spectra. Increased levels of PME have been hypothesized to be associated with intensified cell membrane synthesis, cellular growth, cell nutritional state and rate of cell replication. Several studies identified increased PME signals as a possible diagnostic marker for tumors. PME/PDE ratios were suggested to represent altered relative rates of membrane synthesis, catabolism and metabolic turnover[36,60,61]. Phospholipid derivatives, particularly PCho and PEtn, contribute to the PME resonance[61]. PCho and PEtn resonate at 5.88 ppm and 6.78 ppm respectively.

Liver tumors and metastases

***In vitro* high field ^{31}P MR spectroscopy of liver:** Many *in vitro* ^{31}P MRS animal studies and several *in vitro* ^{31}P MRS studies on human hepatic tumor tissues have been performed. Obtaining a fully representative human hepatic tissue sample for ^{31}P MRS is difficult as the surgical removal and extraction usually results in a period of ischemia/hypoxia, which affects metabolic processes resulting in decreased ATP and increased Pi levels. Nevertheless, *in vitro* ^{31}P MRS may be used to study signals in the PME and PDE peaks that are still unresolved in *in vivo* ^{31}P MR spectra, as these are less affected by short periods of hypoxia.

Bell *et al*[50] investigated the metabolic changes arising in hepatic tumors and the possible systemic effects of these tumors on the liver as a whole. Ten biopsy specimens were obtained from hepatic tumors (one cystadenoma, four hepatocellular carcinomas, four metastatic colonic adenocarcinomas and one metastatic squamous cell carcinoma from the lungs). Five histologically proven normal tissue samples from the same tumor-bearing hepatic lobe were obtained immediately after the blood supply had been clamped and before partial hepatectomy. Six control samples were obtained from morphologically normal liver tissue from patients with histologically proven chronic pancreatitis and known to be free of any hepatic malignancy. Both ^{31}P spectra with proton decoupling and ^{1}H MR spectra with partially water suppression were acquired using high-resolution 11.7T systems. Betaine is the most prominent resonance in the *in vitro* rat liver ^{1}H spectrum but no resonance for betaine was observed in any of the human biopsy samples, suggesting that its presence is species related. The *in vitro* ^{31}P MRS spectrum showed that over 10 different compounds contributed to the PME resonance. The five principal resonances were: PCho, PEtn, glucose-6-phosphate, AMP, and glycerol-3-phosphate. The PDE region included at least 3 different compounds. The two main components were: GPCho and GPEtn. Compared to control tissue, tumor tissue showed significantly lower concentrations of GPCho (0.59 ± 0.15 *vs* 2.46 ± 0.37) and GPEtn (0.57 ± 0.17 *vs* 2.25 ± 0.46), and elevated levels of PCho (1.36 ± 0.50 *vs* 0.17 ± 0.11) and PEtn (2.47 ± 0.84 *vs* 0.16 ± 0.10). It was suggested that the increase in PME/NTP observed in *in vivo* spectra of HCC and

Table 1 Number of different cases

	High PME	High PDE	High Pi	Low PCr
HCC	91% (of 11 cases)	75% (of 4 cases)	0% (of 4 cases)	100% (of 11 cases)
Liver metastasis	100% (of 7 cases)	33% (of 6 cases)	17% (of 6 cases)	100% (of 4 cases)
Liver lymphoma	100% (of 6 cases)	17% (of 6 cases)	100% (of 6 cases)	100% (of 6 cases)

"High" and "low" levels are relative to the amount in normal or benign tissue (from Negendank[70]). PME: Phosphomonoesters; PDE: Phosphodiesters; Pi: Inorganic phosphate; PCr: Phosphorylated creatine; HCC: Hepatocellular carcinoma.

liver metastasis (see next section) is due to increased levels of PCho and PEtn. The decrease in concentration in GPC and GPE observed in this study might be responsible for the change in PDE/NTP seen in *in vivo* spectra. However, the underlying cause of these changes remains partially unknown and requires further study.

Bell *et al*[50] also observed that spectra from histologically normal tissue from the liver tumor-bearing hepatic lobe contained more PCho (0.32 ± 0.18 *vs* 0.17 ± 0.06) and PEtn (0.34 ± 0.12 *vs* 0.16 ± 0.07) than spectra obtained from control tissue. The levels of GPC and GPE showed no significant change.

Previously, in 1993 Dagnelie *et al*[62] studied liver metabolic changes in rats bearing subcutaneous Dunning prostate tumors by *in vivo* and *in vitro* ^{31}P MRS. Although absence of metastatic tumor cells in the liver of all tumor-bearing animals was confirmed by histological examination, hepatic phosphorylation status, phospholipid metabolism, and gluconeogenesis was significantly affected in the tumor-bearing animals. Dagnelie *et al*[63] also investigated liver metabolism in humans with metastatic cancer without evidence of liver metastases by ^{31}P *in vivo* MRS. They included 23 cancer patients and 12 healthy subjects and found markedly elevated PME and reduced PDE levels in the non-metastatic liver compared to controls.

Thus this may complicate the use of increased PME (PCho and PEtn) levels as a sole diagnostic biomarker to detect (metastatic) liver cancer. In addition, as no significant differences between HCC and liver metastasis were observed in biopsy samples by *in vitro* ^{31}P MRS[50,64], the use of *in vivo* ^{31}P MRS seems limited in this differentiation.

***In vivo* ^{31}P MRS of the liver:** In 1985 Maris *et al*[65] combined the results of *in vivo* and *in vitro* ^{31}P MR spectroscopy studies to compare the spectral characteristics of the liver of 2 children, one infant with neuroblastoma stage Ⅳ-S and the other with neuroblastoma stage Ⅳ disease. The ^{31}P MR spectra from the primary tumor in the latter infant, and the spectra from the infiltrated liver regions in both the infants showed substantially elevated PME/β-NTP ratios compared with a spectrum from a normal control. The ratio increased during periods of rapid progression and persisted until treatment became effective. The PME/β-NTP ratio decreased to normal values, during either spontaneous or therapy-induced regression of the disease[66]. It was suggested that the increased PME (corresponding to PEtn and PCho) was due to the need for increased phospholipid synthesis in these tissues. This study demonstrated that ^{31}P MRS could be used to detect tumors and to monitor their response to treatment.

Dixon *et al*[67,68] studied whether hepatic involvement in lymphoma produced biochemical changes that could be detected by *in vivo* ^{31}P MRS of the liver. Twenty-two patients were included. Lymph node biopsies showed that eight patients had Hodgkin's disease and 14 non-Hodgkin's lymphoma. Eleven patients of these 14 had high grade lymphoma and three low grade disease. Six patients, diagnosed with lymphomatous infiltration of the liver on the basis of liver function tests and either ultrasound or CT imaging, had a significantly higher PME/Pi ratio (1.43 ± 0.37) or PME/ATP ratio (0.94 ± 0.27) compared to 25 controls (ages 20-50 years) (0.58 ± 0.11 and 0.37 ± 0.10, respectively) with normal livers. The PME/Pi ratio decreased following chemotherapy to an average of 66% of the initial value (range 40%-82%). In two patients the ratio fell to the normal range and these patients showed clinical remission. Four patients whose liver spectra showed persistently high ratios after therapy died subsequently of progressive disease. *In vitro* ^{31}P MRS studies of extracts of lymphomatous lymph nodes suggested that PEtn was largely responsible for the increased PME signal. In this study, however, no difference was observed between Hodgkin's and non-Hodgkin's lymphoma and between histological grades.

Meyerhoff *et al*[69] used ^{31}P MRS to assess the metabolic state of hepatic cancers and their metabolic response to chemoembolization. Their preliminary report described studies on five patients (two with colon cancer metastasis, two with HCC, and one with adenocarcinoma of unknown primary) and thirteen healthy volunteers. Untreated hepatic tumors showed elevated PME/ATP ratios, reduced ATP and Pi content, and normal PDE levels compared to normal controls. ATP, PME, and/or PDE levels diminished as an acute response to chemoembolization, whereas Pi content increased or stayed relatively constant. This could point to tumor regression and/or necrosis. Long-term follow-up after treatment showed decreased PME/ATP and increased ATP levels, even in the absence of changes on standard imaging. This could be the result of returning normal liver tissue and thus recovery.

Negendank[70] reviewed hundreds of cancer cases in ^{31}P and/or ^{1}H MRS studies that were published up to early 1992. In general he found that human cancers, other than brain, of different types in different locations had similar metabolic characteristics: 173 of 194 cases had high PME and levels, 121 of 166 cases had high PDE levels, 59 of 125 cases had high Pi levels and 96 of 117 cases had low PCr. Table 1 lists the cases with HCC, metastatic liver cancer and lymphoma in the

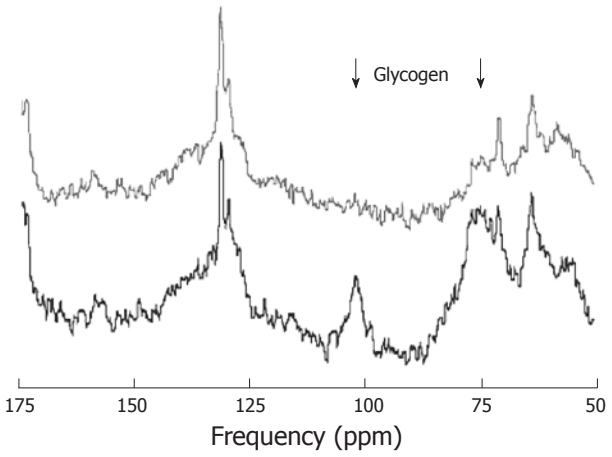

Figure 10 *In vivo* ^{13}C magnetic resonance spectra (1.5T) of human liver tissue obtained from a healthy volunteer before (bottom) and after exercise (top). Resonances of glycogen (101 ppm and around 75 ppm) are reduced after exercise. Other resonances are mainly lipids.

liver. The most frequently used characteristic that differentiated healthy liver tissue from liver lymphoma was an increased PME level, and an increased PME/Pi and increased PME/Pi for HCC, and increased PME/NTP and increased PDE/NTP ratios for metastasis in the liver. Also, an early decrease in PME (or in the PME/PDE ratio) was a good predictor of response to whatever treatment.

Concluding remarks

From these studies it appears that the increase of PME levels is associated with tumor progression and that successful treatment is associated with its decrease. Therefore ^{31}P MRS seems very suitable for treatment response monitoring. Since 2003, however, only a limited number of *in vivo* ^{31}P MRS studies on liver tumors and metastasis have been reported. The reason for this could be the low spatial and time resolution of *in vivo* ^{31}P MRS on 1.5T MR systems. Currently, 3T MR systems have become widely available, dual tuned multi-channel ^{31}P/^{1}H coils have been developed and several techniques, e.g., ^{1}H decoupling, nuclear overhauser enhancement, polarization transfer, have been demonstrated[16] to improve ^{31}P MRS sensitivity and spectral resolution. Finally, high field *in vitro* ^{31}P MRS of cell cultures might establish new markers to distinguish different tumor types and to separate benign from malignant tumors.

^{13}C MR SPECTROSCOPY

As carbon-12 has no net nuclear spin it cannot be detected by MR spectroscopy. In contrast, ^{13}C can be detected by MRS but it has a natural abundance of only 1.11%. Therefore ^{13}C has a relatively low MR sensitivity. In addition, the signal to noise ratio may be negatively affected by ^{1}H coupling. To obtain spectra with acceptable signal to noise it is needed to apply averaging, polarization transfer, and ^{1}H decoupling. The chemical shift dispersion of ^{13}C MRS *in vivo* is large (about 200 ppm) and is also characterized by narrow line widths, resulting in a very good spectral resolution (Figure 10). Although ^{13}C MR spectroscopy is primarily known for MRS of ^{13}C-labeled substrates (e.g., glucose), natural abundance ^{13}C MRS can also be applied.

Metabolites visible in natural abundance ^{13}C MR spectra

Almost all metabolites in the human body contain carbon and therefore in principle a large amount of metabolites can be investigated with ^{13}C MRS, but because of the 1.11% natural abundance this is restricted to only a few highly concentrated compounds such as lipids. Chemical shifts above 150 ppm are indicative of carbonyl groups; carbons adjacent to hydroxyl groups typically resonate in the 60-100 ppm range; CH, CH_2, CH_3 groups resonate in the 45-60 ppm, 25-45 ppm and < 25 ppm ranges, respectively. The resonances of -CH = CH- groups are located around 125 ppm. Dominant lipid resonances are usually found in non-brain tissue. Two distinct resonances at 63 and 73 ppm originate from the glycerol backbone[6].

^{13}C labled metabolites visible in ^{13}C MR spectra

^{13}C MRS with labeled substrates is the only way to study metabolic conversions in the living intact body. Substrates enriched with ^{13}C are usually administrated intravenously to reach a high and stable level in the blood. One of the most commonly used enriched substrate in humans is [1-^{13}C]glucose. Other known substrates are [1,2-$^{13}C_2$]-choline, [1,2-$^{13}C_2$]-ethanolamine, [3-^{13}C]pyruvate and lactate, [2-^{13}C]acetate, [2-^{13}C]glucose and [1,6-$^{13}C_2$]glucose. The latter, often used in studies involving rats and mice, results in two labeled [3-^{13}C]-pyruvate molecules, and thus more signal. Pyruvate is an intermediate common to three major metabolic and catabolic pathways. After i.v. injection, pyruvate is rapidly distributed in the body and taken up by most cells, and then converted into alanine, lactate, or carbon dioxide, depending on the intracellular energy status.

Liver tumors and metastases

Natural abundance ^{13}C MR liver spectrum: In 1983 Bottomley *et al*[71] demonstrated the feasibility of natural abundance ^{13}C MRS at 1.5T. The low sensitivity and the fact that most information could also be obtained from ^{1}H and ^{31}P MRS spectra has prevented widespread application of natural abundance ^{13}C MRS. However, there are areas where it has some advantages such as in the detection of glycogen. Carbohydrate reserves are mainly stored as glycogen in animals and humans, in particular in muscle and liver. Natural abundance ^{13}C MRS detection of glycogen is typically performed *via* the glycogen-C_1 resonance at 100.5 ppm[72]. Some studies on rats have indicated that glycogen levels in hepatic tumors were markedly less than those observed in livers of control animals[73,74].

^{13}C labled ^{13}C MR liver spectrum

^{13}C-labeled glucose: Infusion of enriched ^{13}C-labeled glucose in combination with ^{13}C MRS can provide highly specific information on metabolites and metabolic rates involved in energy metabolism. The ^{13}C-labeled glucose is transported into the cell in the same way as ^{18}FDG used in PET[75]. Where the derivative of ^{18}FDG is trapped inside the cell, indicating areas of high glucose transport and thus indirectly indicating glycolytic activity, the ^{13}C-labeled glucose will enter metabolic pathways like glycolysis and the TCA cycle. This allows the direct study of glucose uptake, the flux through labeled metabolites, relative contributions of glycolytic pathways and oxidative phosphorylation, as well as oxygen consumption[12,76].

^{13}C labeled glucose combined with ^{13}C MRS in human liver tissue is, however, not without difficulties and the number of studies with this technique is still very limited. In 2008 Tomiyasu *et al*[77] monitored liver glycogen synthesis in diabetic patients using ^{13}C MRS on a 3.0T system. The MR signals of liver [1-^{13}C]-glucose and glycogen were assessed and a correlation between the quantity of liver glycogen and the fasting plasma glucose levels was found. To investigate glucose metabolism of liver tumors would be of great interest, but such studies are still lacking.

^{13}C-labeled ethanolamine and choline in experimental tumors: Dixon *et al*[67,78] administrated [^{13}C$_2$]-ethanolamine to mice with lymphomatous liver to study the kinetics of PtdEtn synthesis[79]. The newly synthesized PEtn and PtdEtn could be distinguished from naturally-abundant compounds by their ^{13}C label. The results showed that PtdEtn synthesis in the normal liver largely follows the Kennedy pathway. The data extracted from the ^{13}C MR spectra were fitted to a kinetic model representing this pathway, which allowed them to determine the approximate rates of the various enzymes in the synthetic pathway *in vivo*. They also showed that the overall rate of PtdEtn synthesis from Etn was not increased in lymphomatous liver.

Katz-Brull *et al*[35] investigated the distribution of metabolites following infusion of [1,2-^{13}C]-choline by ^{13}C MRS in mice. In their study MCF7 human breast cancer cells were inoculated s.c. in the right flank of CD-1 female athymic mice. In the tumors significantly more PCho (labeled and unlabeled) was observed than in normal liver and kidney tissue. Therefore, ^{13}C MRS combined with modeling can be used to study choline and ethanolamine metabolism and enzymes rates in mice.

Hyperpolarization and tumor metabolism: Gallagher *et al*[80] performed a ^{13}C MRS *in vitro* study of glutaminase activity in human hepatocellular carcinoma cells using DNP hyperpolarized ^{13}C-labeled glutamine. They showed that the conversion of hyperpolarized [5-^{13}C]glutamine to glutamate senses intramitochondrial glutaminase activity in hepatoma cells. These results represented the first step in the development of an imaging technique for the detection of glutamine metabolism *in vivo*. The rate of glutamine uptake and metabolism to glutamate in HCC cells was shown to be up to 30-fold higher than in normal hepatocytes. And thus this approach has clinical potential such as in the detection of small HCC in the presence of a cirrhotic liver. This study also suggests a new technique to detect changes in tumor cell proliferation in response to cytotoxic treatment since glutamine utilization has been correlated with cell proliferation.

Golman *et al*[75] conducted a metabolic imaging study in P22 tumors implanted on the back of rats. The high signal, due to DNP hyperpolarization, allowed mapping of pyruvate, lactate and alanine in a 5 mm × 5 mm × 10 mm imaging voxel using a 1.5T scanner. Tumor tissue showed a significantly higher lactate content than normal tissue, possibly explained by the Warburg effect. The results indicate that fast noninvasive quantification may be possible. To show that the DNP technique can be used both in small and larger animals, Golman *et al*[81] also conducted nearly similar real-time metabolic imaging studies in rats and pigs. The pig study would provide important information as to whether [1-^{13}C] enriched hyperpolarized dynamics also could be visualized in a more clinically relevant setting. They showed that in both species, where pharmacokinetic parameters widely differ, it is possible to map pyruvate and some of its metabolites in resting skeletal muscle within a clinically useful time frame of about 10 seconds. This indicates the technique may work in humans as well.

Concluding remarks

Hyperpolarization is a promising technique in MR cancer research. Similar images as those in ^{18}FDG-PET can be obtained, but with the further advantages that, without radiation, real (glucose) metabolism is observed. Although this new MR method is far more sensitive than conventional ^{13}C MRS it only allows measurement of single step metabolic conversions associated with rapid cellular uptake of the administered substrate. Therefore, conventional ^{13}C MRS studies will remain valuable to understand and complement results from hyperpolarized ^{13}C MR imaging.

CONCLUSION

Hydrogen is the most commonly studied nucleus, as it has the highest sensitivity compared to ^{31}P and ^{13}C, and essentially can be performed with the same hardware as for standard MR imaging. In liver tumor studies the lactate resonance is related to energy metabolism (Warburg effect) of the tumor. Proton resonances of mobile lipids and the peak of total choline (tCho) have been explored as biomarkers to identify malignant tumors. However, the tCho peak is composed of several unresolved resonances of different choline containing compounds, which makes changes in this signal difficult to interpret.

With ^{31}P MRS phosphorylated choline and ethanolamine containing compounds can be resolved. From

several studies it is known that the increase of PME is associated with tumor progression and that successful treatment is associated with a decrease of PME levels. Therefore ^{31}P MRS could very well be used for treatment response monitoring. Besides the signals from phospholipid metabolism, ^{31}P MR spectra of liver also show signals of ATP and Pi which can be used to investigate tumor energy metabolism.

MRS with ^{13}C as label is a unique method to measure the dynamics of metabolic conversions *in vivo*, but it has hardly been used to examine human liver metabolism due to its technical complexity and relatively low sensitivity. However, developments such as hyperpolarization may open new ways of liver assessment and imaging.

In vivo MR spectroscopy provides a number of adequate research tools to study metabolism in liver tumors and metastasis. However, they are not yet applied often in a clinical setting for diagnosis and treatment monitoring. This may be due to technical challenges associated with the body location of the liver, relatively long scan times needed for a good signal to noise ratio, the need for additional hardware (except ^{1}H MRS) and the need for expertise in spectral interpretation. With higher magnet fields becoming available, new multi-element detection probes, new acquisition techniques for improved spatial and time resolution, better postprocessing, and with new biomarkers it is expected that the research and clinical usefulness of MRS of liver and tumors therein will increase.

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EDITORIAL

Utility of co-transplanting mesenchymal stem cells in islet transplantation

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Abstract

Islet transplantation is characterized by the transplantation of isolated islets from donor pancreata into a diabetic recipient. Although it is a viable choice in the treatment of insulin dependent diabetes mellitus, most patients (approximately 90%) require insulin five years after transplantation. Recently, the co-transplantation of mesenchymal stem cells (MSCs) and islets in animal studies has revealed the effectiveness of MSCs co-transplantation for improving islet function. The mechanisms underlying the beneficial impact of MSCs include immunomodulation and the promotion of angiogenesis. In this review, we discuss MSCs and how they support improved graft survival and function.

Key words: Mesenchymal stem cell; Islet transplantation; Bone marrow; Immunomodulatory; Regulatory T cell; Angiogenesis; Vascular endothelial growth factor; Diabetes mellitus

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INTRODUCTION

According to the International Diabetes Federation (IDF) database, the number of patients with diabetes mellitus (DM) worldwide is 285 million, indicating that 6.4% of the global population have DM. Furthermore, the IDF predict that the number will increase to 438 million by 2030. DM is a serious disease; approximately 4 million people die each year from DM. In addition, DM is a major cause of serious complications such as blindness, renal failure, and ischemic heart disease. Type 1 diabetes is characterized by the irreversible autoimmune destruction of pancreatic β cells and is usually diagnosed in children and young adults[1]. Islet transplantation consists of the transplantation of pancreatic islets that have been isolated from a donor pancreas[2]. The therapeutic effect was regarded as insufficient for a long time; however,

since the use of the "Edmonton Protocol"[2], a markedly improved islet transplant protocol developed at Alberta University, islet transplantation has been performed widely for 10 years. However, according to a recent report, although approximately 70% of patients did not need daily insulin one year after transplantation, approximately 90% of patients required insulin after five years[3]. Therefore, studies aimed at improving the outcome of islet transplantation are still required.

One of the reasons for failure of insulin independence is islet graft loss due to a variety of causes including instant blood-mediated inflammatory reaction[4], acute rejection[5], islet toxicity by immunosuppressive agents[6], and ischemia caused by poor vascularity at transplantation[7] and the embolization effect of the islets[8]. Moreover, islet transplantation also faces the problem of a limited supply of suitable donor human pancreata[9]. Thus, to promote islet transplantation in the future, there is a need to establish a novel donor source and develop more effective treatments to prolong the function of transplanted islets.

WHAT ARE MESENCHYMAL STEM CELLS?

Mesenchymal stem cells (MSCs) are multipotent cells capable of self-renewal and differentiation into a various cell lineages. They are derived from many organs such as bone marrow, adipose tissue, skin, fetal liver, and umbilical cord blood[10,11]. Although the number of MSCs in the bone marrow is very small compared with other component cells (only 0.01%-0.001%)[12], MSCs regulate the maintenance and proliferation of hematopoietic stem cells (HSCs) in the bone marrow[13]. MSCs are able to differentiate into various cells derived from ectoderm (epithelial cells and neurons), mesoderm (connective stroma, cartilage, fat and bone cells), and endoderm (muscle cells, gut epithelial cells, and lung cells)[13]. A number of groups have demonstrated that insulin-producing cells could also be differentiated from MSCs[14-18]. The regeneration of insulin-producing cells is an important theme in research aiming to improve the outcome of cell replacement therapy and has been the focus of some groups. However, in an alternative approach, many studies have examined the effect of transplanting islets with MSCs and have demonstrated improved islet function when co-transplanted with MSCs[7,19-22]. The beneficial effects of MSCs in the context of islet transplantation could be attributed to immunomodulation and angiogenesis.

IMMUNOMODULATORY EFFECT OF MSCS

The pancreata used for clinical islet transplantation are allogeneic and recipients therefore require immunosuppressive drugs to prevent rejection. Recently, Solari *et al*[23] demonstrated that MSCs could promote the prolonged survival of allograft islets in a rat model with limited treatment with immunosuppressive agents. Allogeneic islets transplanted to diabetic rats with syngeneic MSCs survived for over one month whilst islets transplanted alone survived for only seven days. They also demonstrated prolonged graft survival of allogeneic islets transplanted with allogeneic MSCs compared to allogeneic islets transplanted alone. T cell production of interferon (IFN)-γ and tumor necrosis factor (TNF)-α was decreased in allogeneic islets transplanted with syngeneic MSCs. Furthermore, *in vitro* studies of cocultured MSCs and islets generated interleukin (IL)-10 which inhibited $CD4^+$ T cells. Melzi *et al*[20] performed allogeneic islet and allogeneic neural stem cell (NSC) transplantation in a murine model and showed significantly longer graft survival in allogeneic islet/NSC transplanted mice in the absence of immunosuppression, compared to mice transplanted with islets alone (> 100 d survival). Intriguingly, they detected expansion of regulatory T cells in the spleen of co-transplanted mice. These results indicate that MSCs exert an immunomodulatory role and can actively limit the rejection of co-transplanted islets.

The mechanism underlying the immunomodulatory effect of MSCs is likely to be multifactorial and result from the communication between various immune cells and cytokine generation (Figure 1). For example, MSCs can inhibit the proliferation and cytotoxicity of resting natural killer (NK) cells, which are key effector cells of the innate immune system and play an important role in antiviral and anti-tumor immune responses[24]. Spaggiari *et al*[25] demonstrated that the cytokine-induced proliferation of freshly isolated NK cells was inhibited by the presence of MSCs. MSCs also inhibited NK cell activation, cytotoxic activity, and IFN-γ production[26]. These effects are mediated by prostaglandin E_2 (PGE_2) and indoleamine 2,3-dioxygenase (IDO)[13,26].

Another important effect of MSCs is to inhibit the differentiation of monocytes to dendritic cells (DCs) that, following DC maturation, present antigens to naïve T cells[27,28]. MSCs also inhibit TNF-α production by DCs and upregulate IL-10 production by plasmacytoid DCs (pDCs)[29] - effects modulated by PGE_2. These effects of MSCs upon DC function undoubtedly contribute to their anti-inflammatory and immunoregulatory effects.

MSCs may also directly inhibit $CD4^+$ T cells, $CD8^+$ T cells, and B cells, immune cells involved in rejection of allogeneic cells, by releasing soluble mediators, including PGE_2, IDO, or soluble human leukocyte antigen (sHLA)-G5. Inhibition of $CD4^+$ T cells impairs B cell proliferation and antibody production[13]. $CD8^+$ cytotoxic T cells are involved in killing virus-infected or allogeneic cells, and MSCs are capable of inhibiting the induction of $CD8^+$ T cell responses and preventing cytotoxicity[30]. MSCs inhibit B cell proliferation and antibody secretion, as well as their differentiation to plasma cells[31]. On the other hand, MSCs may induce the generation of regulatory T cells, which suppress immune cell activation, and help to maintain homeostasis and promote self tolerance by inducing production of IL-10 from pDCs and by releasing HLA-G5[29,32]. In

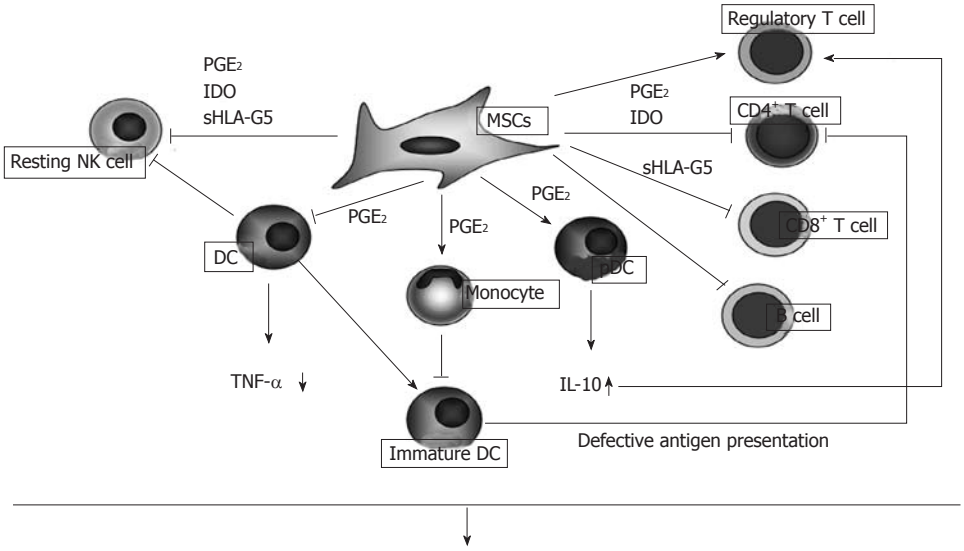

Figure 1 Immunomodulatory effect of mesenchymal stem cells (modified and quoted from Uccelli *et al*[13]). Mesenchymal stem cells (MSCs) can inhibit the proliferation and cytotoxicity of resting natural killer (NK) cells *via* the generation of mediators, including prostaglandin E_2 (PGE_2), indoleamine 2,3-dioxygenase (IDO) and soluble human leukocyte antigen (sHLA)-G5; MSCs inhibit the differentiation of monocyte to antigen presenting dendritic cells (DCs). MSCs also inhibit TNF-α production by DCs and upregulate IL-10 production by plasmacytoid DCs (pDCs): effects modulated by PGE_2; MSCs directly inhibit $CD4^+$ T cell, $CD8^+$ T cell, and B cells that are involved in allogeneic cell rejection by releasing PGE_2, IDO, or sHLA-G5. $CD4^+$ T cell inhibition limits B cell proliferation and antibody production whilst $CD8^+$ T cell inhibition prevents cytotoxicity. MSCs induce generation of immunomodulatory regulatory T cells that suppress immune activation, help to maintain homeostasis, and promote self tolerance by production of IL-10 from pDCs and by releasing HLA-G5. Thus, MSCs can promote immunotolerance and facilitate the engraftment of allogeneic islets.

summary, MSCs can promote immunological tolerance and facilitate the survival and function of allogeneic islets. It is likely, however, that the immunomodulatory roles of MSCs have not been fully clarified.

ANGIOGENIC EFFECT OF MSCS

Pancreatic islets have a rich vascular supply in the pancreas, with some reports indicating that islets receive 5%-10% of pancreatic blood flow, despite the islet mass only comprising 1%-2% of the total pancreas[33,34]. However, isolated islets are avascular, as the process of islet isolation destroys the vascular network between the islet and surrounding tissue[35]. As a result, islets undergo prolonged ischemia during the reconstruction of the vascular network, which may take approximately 14 d[36] and many islets become damaged. It is thus apparent that strategies to limit islet ischemia are necessary to improve the outcome of islet transplantation.

Some studies suggest that angiogenic factors, such as vascular endothelial growth factor-A (VEGF-A) and angiopoietin-1, are required to generate a vascular network around transplanted islets[37,38]. Recently, the pro-angiogenic effects of MSCs have been examined (Figure 2). The process of revascularization consists of proteolytic digestion of the vascular wall and subsequent migration, proliferation, and differentiation of endothelial cells (ECs)[39]. MSCs express platelet-derived growth factor (PDGF) receptors and respond to PDGF production by ECs during revascularization[40]. MSCs promote EC migration by producing proteases that facilitate immature EC sprouting[41] and upregulating the expression of angiopoietin and VEGF in ECs, as these factors promote angiogenesis and stability of the developing vasculature[42]. The roles of MSCs in angiogenesis have been explored in experimental models of ischemia. Martens *et al*[43] demonstrated that MSCs produced VEGF and induced neovascularization in the ischemic myocardium. Jiang *et al*[44] showed that transplantation of MSCs into ischemic limbs promoted angiogenesis. Johansson *et al*[45] explored the enhancement of angiogenesis by MSCs in the context of islets. They first examined cultures of human islets and ECs in the presence or absence of MSCs. The study indicated that the inclusion of MSCs promoted EC proliferation and migration of ECs to the surface of islets to form a "coat"[45]. Islets with this surrounding "coat" of endothelial cells survived for a long time in culture and exhibited improved insulin release[45]. The coated islets had many sprouts and were connected to other endogenous islets by vessel-like structures[45]. These findings indicated that MSCs act to promote EC proliferation in both donor and recipient sides, together with sprout formation and growth of ECs into the islet. Thus, MSCs may exert a potent angiogenic function and contribute to islet engraftment by promoting islet vascularization.

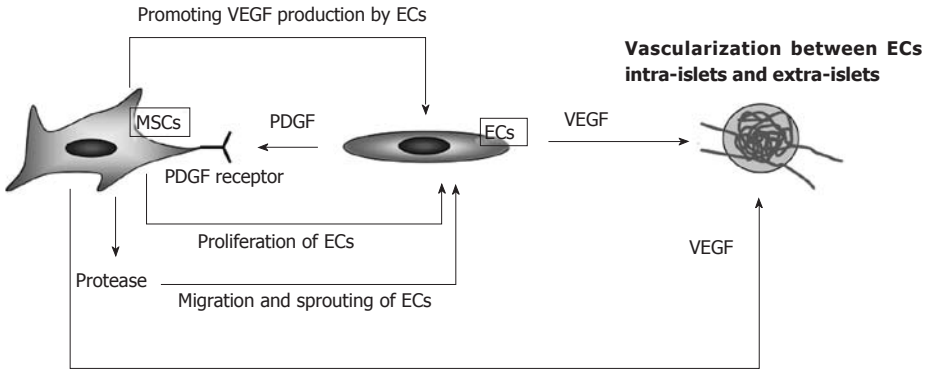

Figure 2 Pro-angiogenic effect of mesenchymal stem cells. The process of revascularization requires proteolytic digestion of the vascular wall and subsequent migration, proliferation, and differentiation of endothelial cells (ECs). Mesenchymal stem cells (MSCs) express platelet-derived growth factor (PDGF) receptors and respond to PDGF production by ECs during revascularization. MSC-derived proteases promote EC migration and immature EC sprouts and upregulate the expression of angiopoietin and vascular endothelial growth factor (VEGF) in ECs, thereby promoting both angiogenesis and vascular stability. MSCs also produce VEGF and induce neovascularization. MSCs promote both donor and recipient EC proliferation, EC sprout formation, the ingrowth of ECs into islets and the formation of a vascular network between intra- and extra-islet vessels.

Recently, Ito *et al*[21] co-transplanted rat islets and MSCs into diabetic severe combined immunodeficiency mice and evaluated the rate of normoglycemia and extent of islet vascularization. All the diabetic mice that received 500 islets with 10^7 MSCs exhibited normoglycemia, compared to 30% of mice transplanted with islets alone. Neovascular density was also increased in the islet/MSCs co-transplanted group and was associated with strong expression of VEGF and endothelial von Willebrand factor. Sakata *et al*[7] and Figliuzzi *et al*[22] also evaluated the impact of MSCs upon transplanted islets, and found a similar improvement of islet function and vascularization. The beneficial impact of vascularization is in accord with our previous work indicating that hyperbaric oxygen therapy prevented cellular apoptosis of islets[46]. These data indicate that promoting islet vascularization by co-transplanting MSCs acts to limit the duration and severity of islet ischemia, thereby limiting islet cell apoptosis and promoting islet integrity and function.

CONCLUSION

It is likely that MSCs may exert beneficial effects in addition to those previously outlined. For example, Olerud *et al*[19] demonstrated that neural crest stem cells, a kind of MSCs, augment islet cell proliferation and improve islet function. In addition, Melzi *et al*[20] showed that transplanted bone marrow cells stimulated pancreatic β-cell proliferation after streptozotocin-induced pancreatic injury.

In conclusion, MSCs may exert beneficial immunomodulatory and pro-angiogenic effects when co-transplanted with islets. Immunomodulatory effects include the functional inhibition of immunocompetent cells, such as NK cells, DCs, cytotoxic T cells, and B cells. MSCs may also induce the generation of regulatory T cells that promote immunological tolerance. The pro-angiogenic effects of MSCs result from the release of angiogenic factors and promotion of the vascular network linking islets to the surrounding tissue. This pro-angiogenic role limits the duration and severity of islet ischemia and improves islet function. We therefore believe that the co-transplantation of MSCs represents a viable method for improving islet transplantation. Recently, a clinical trial of combined islet and hematopoietic stem cell allotransplantation was performed[47]. The data did not support the effectiveness of HSCs co-transplantation in prevention of graft rejection and in avoiding side effects of immunosuppression. Moreover, Melzi *et al*[20] reported the risk of inducing cancer because of NSC co-transplantation, as well as proving the effectiveness of this strategy to prevent islet graft rejection. Thus, further studies examining the effect of MSCs in a clinical setting should be undertaken.

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ORIGINAL ARTICLE

Helicobacter pylori's virulence and infection persistence define pre-eclampsia complicated by fetal growth retardation

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Abstract

AIM: To better understand the pathogenic role of *Helicobacter pylori* (*H. pylori*) in pre-eclampsia (PE), and whether it is associated or not with fetal growth retardation (FGR).

METHODS: Maternal blood samples were collected from 62 consecutive pregnant women with a diagnosis of PE and/or FGR, and from 49 women with uneventful pregnancies (controls). Serum samples were evaluated by immunoblot assay for presence of specific antibodies against *H. pylori* antigens [virulence: cytotoxin-associated antigen A (CagA); ureases; heat shock protein B; flagellin A; persistence: vacuolating cytotoxin A (VacA)]. Maternal complete blood count and liver enzymes levels were assessed at delivery by an automated analyzer.

RESULTS: A significantly higher percentage of *H. pylori* seropositive women were found among PE cases (85.7%) compared to controls (42.9%, $P < 0.001$). There were no differences between pregnancies complicated by FGR without maternal hypertension (46.2%) and controls. Importantly, persistent and virulent infections (VacA/CagA seropositive patients, intermediate leukocyte blood count and aspartate aminotransferase levels) were exclusively associated with pre-eclampsia complicated by FGR, while virulent but acute infections (CagA positive/VacA negative patients, highest leukocyte blood count and aspartate aminotransferase levels) specifically correlated with PE without FGR.

CONCLUSION: Our data strongly indicate that persistent and virulent *H. pylori* infections cause or contribute to PE complicated by FGR, but not to PE without feto-placental compromise.

Key words: *Helicobacter pylori*; Virulence factors; Pre-eclampsia; Fetal growth retardation; Cytotoxin-associated antigen A; Vacuolating cytotoxin A

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INTRODUCTION

Pre-eclampsia (PE) is a severe hypertensive pregnancy-re-

lated disorder that affects 5%-8% of women worldwide, thus representing the main cause of feto-maternal mortality and morbidity[1,2]. PE is often associated with fetal growth retardation (FGR), defined as failure of the fetus to achieve its genetically determined growth potential[3,4]. FGR is commonly considered a severe complication of PE, but whether or not PE and FGR are manifestations of the same disorder, or two distinct pathologies, still remains unclear.

PE is characterized by excessive maternal inflammatory response, with high circulating levels of pro-inflammatory cytokines and endothelial injury[1,2]. Despite being an object of intense investigation, the etiopathogenetic mechanisms of PE are still poorly understood. Several lines of evidence suggest that subclinical infections could play a role in the onset of PE[5,6].

We previously reported a strong association between *Helicobacter pylori* (*H. pylori*) infection and PE[7]. *H. pylori* is a Gram-negative bacterium responsible for the large majority of peptic ulcers, gastric cancer, and gastric mucosa-associated lymphoid tissue lymphoma[8]. It has been demonstrated that this pathogen enhances platelets activation and thrombus formation[9,10], thus inducing endothelial inflammation and injury. Therefore, *H. pylori* could directly cause or intensify the generalized inflammation and endothelial dysfunction typical of PE[7]. Furthermore, it was recently observed that *H. pylori* seropositive PE subjects are characterized by a more severe inflammatory status[11] and lipid peroxidation[12].

The role of cytotoxin-associated antigen A (CagA) in inducing a severe immunogenic response in patients infected by *H. pylori* is now well established[13]. Nevertheless, other virulence factors could be involved in the severe inflammatory response mediated by this bacterium. The vacuolating cytotoxin A (VacA) is a protein produced by *H. pylori* with several effects on vulnerable cells, such as vacuolation with alteration of the endo-lysosomal function and mitochondrial damage accompanied by cytochrome C release and apoptosis[14].

Ureases allow colonization of the gastric mucosa by catalyzing the hydrolysis of urea and help to recruit neutrophils and monocytes in the mucosa, thus inducing pro-inflammatory cytokines production[15].

Heat shock protein B (HspB) has been shown to increase the risk of gastric carcinoma, by directly inducing hyper-proliferation of gastric cells[16]. Moreover, it strongly activates the immune system and stimulates a massive immune response in patients with gastritis and gastric cancer[17-19].

To better understand the pathogenic role of *H. pylori* in pre-eclampsia, we investigated maternal serum positivity for antibodies against CagA, VacA, HspB, ureases A, C, E and H (UreA, UreC, UreE, UreH), and for flagellin A (FlagA). FlagA is the major *H. pylori* flagellin isoform, mainly expressed during late exponential growth phase and represents a good *H. pylori* virulence index[20].

To correlate *H. pylori* virulence with PE severity, and to detect differences in *H. pylori* profiles between PE and FGR pregnancies, we determined seropositivity for the above mentioned antigens in three populations: PE without FGR, PE complicated by FGR, and FGR without PE.

Finally, we verified the reported association between *H. pylori* infection and elevated leukocyte blood count and serum amino-transferases levels[21].

MATERIALS AND METHODS

Population and samples

The study was approved by our Hospital Ethics Committee "Comitato Etico Interaziendale AA.OO O.I.R.M./S.Anna di Torino and Ordine Mauriziano di Torino" and written informed consent was obtained from each participating woman.

Maternal blood samples (5 mL) were collected before delivery from 62 consecutive pregnant women with diagnosis of PE and/or FGR, and from 49 women with normotensive pregnancies with normal fetal growth and normal uterine and umbilical Doppler flow velocimetry (FVW).

PE was diagnosed when hypertension (systolic blood pressure $\geqslant$ 140 mmHg or diastolic blood pressure $\geqslant$ 90 mmHg) and proteinuria ($\geqslant$ 300 mg/24 h) appeared after 20 wk of gestational age in previously normotensive women, according to the American College of Obstetricians and Gynecologists criteria[22]. PE was considered severe when one or more of the following criteria were present: systolic pressure $\geqslant$ 160 mmHg or diastolic pressure $\geqslant$ 110 mmHg on two occasions at least 6 h apart, or significant proteinuria ($\geqslant$ 3 + on urine dipstick or > 5 g in a 24-h urine)[22]. Patients with PE were further classified as either having early-onset ($\geqslant$ 34 wk), or late-onset (> 34 wk) disease according to the gestational age of PE diagnosis.

The hemolysis-elevated liver enzymes-low platelets (HELLP) syndrome was defined by the following criteria: hemolysis (characteristic peripheral blood smear and serum lactate dehydrogenase $\geqslant$ 600 U/L), elevated liver enzymes (serum aspartate aminotransferase $\geqslant$ 70 U/L), and low platelet count (< 100 000/μL)[23].

The diagnosis of FGR was made according to the following criteria: ultrasound measurement of fetal abdominal circumference below the 10th centile[24] or growth velocity below the 10th percentile[25] and/or birth weight below the 10th centile, according to Italian reference values[26] with abnormal umbilical arteries Doppler FVWs[27] and/or abnormal uterine artery Doppler FVWs (resistance index of > 0.58)[28]. Exclusion criteria were: multiple pregnancies, congenital malformations, and prenatal or postnatal diagnosis of chromosomal anomalies in number and/or structure.

For all cases and controls, the following data were collected: maternal age at delivery, gestational age at birth, week of PE onset, mode of delivery, neonatal sex, birth weight, placental weight, parity, blood pressure, urinary protein, complete blood count and differentials (count and percentage of neutrophils, lymphocytes, monocytes, eosinophils, and basophils), liver enzymes levels, risk factors for PE (previous pregnancy with PE, autoim-

Table 1 Clinical characteristics of study populations (continuous variables)

Variable	Controls (n = 49) Median (25th-75th)	PE-only (n = 17) Median (25th-75th)	PE FGR (n = 32) Median (25th-75th)	FGR-only (n = 13) Median (25th-75th)	P value[1]
Maternal age at delivery (yr)	30 (28-33)[4]	29 (24-32)	30 (26-34)[7]	25 (24-26)[4,7]	[4]< 0.001; [7]0.002
Gestational age at delivery (wk)	40 (39-41)[2,3,4]	30 (28-31)[2,5,6]	32 (31-34)[3,5]	34 (32-39)[4,6]	[2,3,4]< 0.001; [5]0.045; [6]0.009
Neonatal weight (g)	3380 (3170-3700)[2,3,4]	1140 (1045-1570)[2]	1278 (920-1668)[3]	1600 (1060-2730)[4]	[2,3,4]< 0.001
Placental weight (g)	600 (500-650)[2,3,4]	300 (240-410)[2]	280 (215-360)[3]	345 (300-470)[4]	[2,3,4]< 0.001
Systolic blood pressure (mmHg)	120 (110-120)[2,3]	160 (150-160)[2,6]	150 (148-160)[3,7]	120 (120-125)[6,7]	[2,3,6,7]< 0.001
Diastolic blood pressure (mmHg)	75 (70-80)[2,3]	100 (100-100)[2,6]	100 (95-105)[3,7]	77 (75-80) [6,7]	[2,3,6,7]< 0.001
Proteinuria (g/24 h)	0 (0-0)[2,3]	2.21 (1.52-3)[2,6]	1.34 (0.79-2.38)[3,7]	0 (0-0)[6,7]	[2,3,6,7]< 0.001

[1]P values were calculated by non-parametric Kruskal-Wallis H test, with post-hoc analysis by Mann-Whitney U test. [2]Comparison between controls and PE-only group; [3]Comparison between Controls and PE FGR group; [4]Comparison between controls and FGR-only group; [5]Comparison between PE-only and PE FGR groups; [6]Comparison between PE-only and FGR-only groups; [7]Comparison between PE-only and FGR-only groups. PE: Pre-eclampsia; FGR: Fetal growth retardation.

mune diseases, diabetes, cardiovascular diseases, or other common risk factors for PE), and family history of pre-eclampsia and/or cardiovascular diseases.

Venous blood samples were collected into Vacutainer tubes (Becton Dickinson, Plymouth, United Kingdom) without anticoagulant. Serum was separated by centrifugation immediately after clotting and stored at -30 ℃ until assayed.

Serology

Serum samples were evaluated for specific antibodies against *H. pylori* antigens by commercially available Heli-Blot assay (Nurex; Sassari, Italy). *H. pylori* seropositivity was determined according to manufacturer instructions. Briefly, diluted serum samples (1:100) were incubated with Heli-Blot strips for 30 min. The strips were then incubated consecutively with anti-IgG for 30 min, with substrate for range of minutes, and then dried. Results were read according to the standard control protein bands provided. The standard *H. pylori* antigens available in the strip included: 120 kDa (CagA), 89 kDa (VacA), 60 kDa (Urease C), 54 kDa (HSP), 35 kDa (Flagellin), 30 kDa (Urease H), 26 kDa (Urease A), and 19 kDa (Urease E). The presence of one of the three most specific antigens (CagA, VacA, or flagellin) or the presence of two of the three smallest antigens was considered a positive test for the diagnosis of *H. pylori* infection.

Statistical analysis

Data analysis was performed using SPSS version 17.0 (SPSS Inc., Chicago, Illinois, United States). Continuous variables were reported as medians and interquartile ranges (25th-75th percentiles). Medians among groups were analyzed by non-parametric Kruskal-Wallis H test, with post-hoc analysis by Mann-Whitney U test. Categorical variables are presented as frequencies (percentages) and the comparison between different groups was done with a χ^2 test by means of a 2 × 2 contingency table; Fisher's exact test was used for small sample sizes. All tests were 2-tailed and results were considered significant for a P value less than 0.05. The odds ratios (OR) and 95% confidence intervals (CI), adjusted for maternal age at delivery, pre-pregnancy body mass index, parity, presence of maternal and family risk factors, were calculated using logistic regression analysis to assess the risk of PE and/or FGR associated with *H. pylori* infection.

RESULTS

Population

A total of 111 serum samples from pregnant women were examined: 49 uneventful pregnancies (Ctrl) and 62 pathological pregnancies complicated by fetal growth retardation (FGR-only, n = 13), pre-eclampsia (PE-only, n = 17), or both (PE-FGR, n = 32). Characteristics of the study population are summarized in Tables 1 and 2.

We found that normotensive women with pregnancy complicated by FGR were significantly younger (median of 25 years with an interquartile range of 24-26 years) compared to controls and PE women (both with a median age of 30 years). As expected, pregnancies complicated by PE and/or FGR were delivered more often by caesarean section. Moreover, pathological cases led to lower neonatal and placental weight compared to controls, due to lower gestational age at delivery and reduced fetal growth.

Pre-eclamptic mothers presented higher blood pressure values and urine protein concentrations. The presence of family risk factors was increased in PE cases without FGR (Table 2), while maternal risk factors for PE did not differ among groups (Table 2). The percentage of nulliparous women was significantly higher in the PE group than in controls. In 45 PE mothers, hypertension and proteinuria appeared early (before 34 wk) and in 32 of them these symptoms were severe; moreover five PE pregnancies were complicated by HELLP syndrome.

Leukocyte blood count, platelet count, and serum amino-transferases values in normal and pathologic pregnancies

Pre-eclamptic pregnancies were characterized by significantly higher values of total leukocyte count (P = 0.004) and serum amino-transferases [alanine aminotransferase

Table 2 Clinical characteristics of study populations (categorical variables) *n* (%)

Variable		Controls (*n* = 49)	PE-only (*n* = 17)	PE FGR (*n* = 32)	FGR-only (*n* = 13)	*P* value[5]
Cesarean section delivery		15 (30.6)[6,7,8]	16 (94.1)[6]	29 (90.6)[7]	9 (69.2)[8]	[6,7]< 0.001; [8]0.022
Neonatal sex	Male	19 (38.8)[7]	7 (41.2)	20 (62.5)[7]	6 (46.2)	[7]0.043
	Female	30 (61.2)	10 (58.8)	12 (37.5)	7 (53.8)	NS
Nulliparae		31 (63.3)[6,7]	16 (94.1)[6]	27 (84.4)[7]	10 (76.9)	[6]0.015; [7]0.047
Maternal risk factors		4 (8.2)	2 (11.8)[1]	8 (25.0)	1 (7.7)	NS
	Autoimmune diseases	1 (2.0)	2 (11.8)	4 (12.5)	0 (0)	NS
	Cardiovascular diseases	3 (6.1)	1 (5.9)	4 (12.5)	1 (7.7)	NS
Family risk factors		20[2] (40.8)[6]	12[3] (70.6)[6,10]	13[4] (40.6)	2 (15.4)[10]	[6]0.049; [10]0.004
	Hypertension	9 (18.4)	8 (47.1)	10 (31.3)	2 (15.4)	NS
	Diabetes	10 (20.4)	3 (17.6)	5 (15.6)	0 (0)	NS
	Cardiovascular diseases	5 (10.2)	2 (11.8)	3 (9.4)	0 (0)	NS
Other complications:	FGR	0 (0.0)[7,8]	0 (0.0)[9,10]	32 (100)[7,9]	13 (100)[8,10]	[7,8,9,10]< 0.001
	Early onset PE	-	16 (94.1)	29 (90.6)	-	NS
	Severe PE	-	13 (76.5)	19 (59.4)	-	NS
	HELLP syndrome	-	3 (17.6)	2 (6.3)	-	NS

[1]One patient presented both maternal risk factors (autoimmune and cardiovascular diseases); [2]Four patients presented two family risk factors (3 hypertension and diabetes; 1 diabetes and cardiovascular disease); [3]One patient presented two family risk factors (hypertension and diabetes); [4]Five patients presented two family risk factors (4 hypertension and diabetes; 1 hypertension and cardiovascular disease). [5]*P* values were calculated by chi-square test (χ^2). [6]Comparison between controls and PE-only group; [7]Comparison between controls and PE FGR group; [8]Comparison between controls and FGR-only group; [9]Comparison between PE-only and PE FGR groups; [10]Comparison between PE-only and FGR-only groups. NS: Non significant; PE: Pre-eclampsia; FGR: Fetal growth retardation; HELLP: Hemolysis-elevated liver enzymes-low platelets.

(ALT), aspartate aminotransferase (AST) $P = 0.006$ and $P = 0.029$, respectively], while eosinophil count and percentage were significantly lower ($P = 0.028$ and $P = 0.02$, respectively) compared to controls. However, if we exclude pathological cases complicated by HELLP syndrome, only ALT levels remained significantly higher in PE (Table 3). Normotensive pregnancies complicated by FGR showed significantly higher leukocyte levels compared to controls ($P = 0.045$, Table 3). Moreover, the FGR-only lymphocyte percentage was significantly higher relative to PE-only ($P = 0.047$, Table 3).

H. pylori seropositivity was increased in PE-FGR but not in FGR-only pregnancies

H. pylori seropositivity was significantly more frequent in PE women with or without FGR (85.7%) ($P < 0.001$; OR 9.22, 95% CI: 2.83-30.04), while it did not differ between FGR-only (46.2%) and controls (42.9%) (Table 4, Figure 1A). Further subdivision of PE group showed a higher prevalence of seropositive subjects among PE-FGR cases (93.8%) ($P < 0.001$; OR 35.56, 95% CI: 5.22-242.43) compared to controls; while in the PE-only group, the percentage of *H. pylori* seropositive women was higher, but not statistically significant (70.6%), relative to controls (Table 4, Figure 1A).

CagA and VacA seropositivity was increased in pre-eclamptic but not in FGR-only pregnancies

Similar to *H. pylori* seropositivity, the presence of antibodies against CagA antigen was prevalent only in PE pregnant women (81.6%) relative to controls (22.4%) ($P < 0.001$; OR 17.66, 95% CI: 5.25-59.49), while there were no differences between FGR-only cases (38.5%) and controls (Table 4, Figure 1B). CagA seropositivity was significantly more frequent in both PE-FGR (90.6%) ($P < 0.001$; OR 54.97, 95% CI: 9.24-326.88) and PE-only groups (64.7%) ($P = 0.038$; OR 5.20, 95% CI: 1.09-24.69), relative to controls. VacA seropositivity was significantly higher in PE-FGR cases (87.5%) ($P < 0.001$; OR 19.64, 95% CI: 3.75-102.98), while there were no differences between PE-only (55.6%) and FGR-only cases (53.8%), relative to controls (40%) (Table 4, Figure 1C).

Seropositivity for both CagA and VacA antibodies was associated with higher risk of PE-FGR (OR 45.44; 95% CI: 7.79-265.18). In fact, 87.5% of PE-FGR pregnancies were CagA and VacA seropositive, compared to 22.4% in Ctrl group (Table 5, Figure 1D). Patients seropositive for VacA, but not for CagA, were nine controls (18.4%), one FGR (7.7%), and no PE women, while seropositivity for CagA only was a specific feature of the PE-only group (Table 5). Seronegative women for both anti-CagA and VacA antibodies were only 9.4% in the PE-FGR group, while they were 59.2%, 35.3% and 53.8% in the Ctrl, PE-only and FGR-only groups, respectively (Table 5, Figure 1D). Importantly, CagA and VacA seronegativity was associated with a lower risk of developing pre-eclampsia complicated by fetal growth retardation (OR 0.04; 95% CI: 0.01-0.22).

UreC and UreE seropositivity was higher in PE-FGR pregnancies

We found significantly higher UreC and UreE seropositivity in PE-FGR patients (46.9%; $P = 0.018$ and 56.3%; $P = 0.003$, respectively) relative to controls (26.5% and 24.5%, respectively) (Figure 2B and C), while there were no differences among groups for HspB, FlagA, UreA, and UreH (Table 4, Figure 2A and D-F). Odds ratios calculation showed higher risk of developing PE-FGR in

Table 3 Leukocytes, platelets and liver enzymes in normal and pathological pregnancies

Variable	Normal values in Italian female population range	Controls (n = 49) Median (25th-75th)	All PE (n = 49) Median (25th-75th)	PE-only (n = 17) Median (25th-75th)	PE FGR (n = 32) Median (25th-75th)	FGR-only (n = 13) Median (25th-75th)	P value[2]
Total leukocyte count ($1 \times 10^3/\mu L$)	4.00-11.00	10.56 (9.21-11.65)[3,7,5,6]	12.03 (10.69-14.1)[3]	12.34 (10.71-13.83)[5]	11.83 (10.2-14.51)[6]	12.27 (11.21-13.47)[7]	[3]0.004; [7]0.045 [5]0.007; [6]0.024
Neutrophils ($1 \times 10^3/\mu L$)		8.27 (7.45-9.19)	10.09 (7.30-11.6)	10.15 (7.81-12.10)	9.92 (7.30-11.51)	9.34 (6.27-9.48)	NS
(%)	45.0-73.0	75.8 (68.1-78.8)	76.7 (68.37-87.1)	80 (72.5-90.1)	72.1 (68.2-83.6)	64.4 (57.3-77.3)	NS
Lymphocytes ($1 \times 10^3/\mu L$)		2.02 (1.68-2.26)	2.08 (1.3-3.06)	1.86 (1.22-2.34)	2.08 (1.31-3.07)	3.15 (2.13-3.79)	NS
(%)	19.0-47.0	18.7 (14-23.5)	17.4 (10.8-22.43)	14.55 (8.9-18.15)[8]	18.8 (11.1-23.2)	25.9 (17.4-34.3)[8]	[8]0.047
Monocytes ($1 \times 10^3/\mu L$)		0.54 (0.47-0.74)	0.59 (0.3-0.85)	0.61 (0.25-0.81)	0.58 (0.3-0.88)	0.89 (0.54-1.05)	NS
(%)	3.0-9.0	5.4 (4.5-6)	5.3 (3-7.3)	4.15 (2.15-7.75)	5.4 (3.5-7.3)	6.1 (4.9-7.9)	NS
Eosinophils ($1 \times 10^3/\mu L$)		0.15 (0.09-0.19)[3]	0.05 (0.02-0.1)[3]	0.04 (0.02-0.11)	0.05 (0.03-0.1)	0.17 (0.03-0.33)	[3]0.028
(%)	0.2-4.4	1.2 (0.9-1.8)[3]	0.5 (0.2-0.8)[3]	0.35 (0.1-0.8)	0.5 (0.2-0.8)	1.7 (0.2-2.4)	[3]0.020
Basophils ($1 \times 10^3/\mu L$)		0.02 (0.01-0.03)	0.02 (0.01-0.03)	0.03 (0.01-0.03)	0.02 (0.01-0.03)	0.01 (0-0.04)	NS
(%)	0.1-1.3	0.2 (0.1-0.3)	0.2 (0.1-0.3)	0.2 (0.15-0.25)	0.2 (0.1-0.3)	0.1 (0-0.3)	NS
Platelets ($1 \times 10^3/\mu L$)	150-400	219 (170-240)[5]	187 (126-228)	170 (111-214)[5,8]	191 (143-234)	235 (177-285)[8]	[5]0.024; [8]0.031
Platelets[1] ($1 \times 10^3/\mu L$)	150-400	219 (170-240)	191 (161-234)	180.5 (154-228)	191 (165-242)	235 (177-285)	NS
ALT (U/L)	< 34	15 (10-19)[3,5]	23 (14-46)[3]	26 (19-150)[5,8]	19.5 (13.5-35)	14 (10-21.5)[8]	[3]0.006; [5]0.002; [8]0.026
ALT[1] (U/L)	< 34	15 (10-19)[3]	20 (14-31)[3]	25 (15-46)	18 (13-27)	14 (10-21.5)	[3]0.023
AST (U/L)	< 31	17.5 (14-19)[3]	21 (16-39)[3,4]	25 (16-123)[8]	20 (16-35.5)[9]	14 (12-18)[4,8,9]	[3]0.029; [4]0.018; [8]0.031; [9]0.026
AST[1] (U/L)	< 31	17.5 (14-19)	19 (15.5-33)	19 (15-39)	18.5 (16-32)	14 (12-18)	NS

[1]Hemolysis-elevated liver enzymes-low platelets cases excluded; [2]P values were calculated by non-parametric Kruskal-Wallis H test, with post-hoc analysis by Mann-Whitney U test. [3]Comparison between controls and all PE group; [4]Comparison between all PE and FGR-only groups; [5]Comparison between controls and PE-only group; [6]Comparison between controls and PE FGR group; [7]Comparison between controls and FGR-only group; [8]Comparison between PE-only and FGR-only groups; [9]Comparison between PE-only and FGR-only groups. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; NS: Non significant; PE: Pre-eclampsia; FGR: Fetal growth retardation.

Table 4 Seropositivity against *Helicobacter pylori*, cytotoxin-associated antigen A, vacuolating cytotoxin A, ureases A, C, E and H, heat shock protein B and flagellin A n (%)

	Controls (n = 49)	All PE (n = 49)	PE-only (n = 17)	PE FGR (n = 32)	FGR-only (n = 13)	P value[1,2]	Odds ratio[1] (95% CI)
Helicobacter pylori	21 (42.9)[3,5]	42 (85.7)[3]	12 (70.6)	30 (93.8)[5]	6 (46.2)	[3,5]< 0.001	[3]9.22 (2.83-30.04) [5]35.56 (5.22-242.43)
CagA	11 (22.4)[3,4,5]	40 (81.6)[3]	11 (64.7)[4]	29 (90.6)[5]	5 (38.5)	[3,5]< 0.001 [4]0.038	[3]17.66 (5.25-59.49) [4]5.20 (1.09-24.69) [5]54.97 (9.24-326.88)
VacA	20 (40.8)[3,5]	37 (75.5)[3]	9 (52.9)	28 (87.5)[5]	6 (46.2)	[3]0.005 [5]< 0.001	[3]4.89 (1.62-14.73) [5]19.64 (3.75-102.98)
HspB	15 (30.6)	21 (42.9)	5 (29.4)	16 (50.0)	6 (46.2)	NS	
FlagA	13 (26.5)	22 (44.9)	6 (35.3)	16 (50.0)	5 (38.5)	NS	
UreA	10 (20.4)	13 (26.5)	4 (23.5)	9 (28.1)	3 (23.1)	NS	
UreC	13 (26.5)[3,5]	19 (38.8)[3]	4 (23.5)	15 (46.9)[5]	4 (30.8)	[3]0.042 [5]0.018	[3]2.84 (1.04-7.75) [5]4.02 (1.27-12.80)
UreE	12 (24.5)[3,5]	26 (53.1)[3]	8 (47.1)	18 (56.3)[5]	3 (23.1)	[3]0.004 [5]0.003	[3]4.41 (1.59-12.26) [5]6.29 (1.88-21.04)
UreH	8 (16.3)	13 (26.5)	5 (29.4)	8 (25.0)	4 (30.8)	NS	

[1]Adjusted for maternal age at delivery, pre-pregnancy body mass index, parity, and presence of maternal and family risk factors; [2]P values were calculated by χ^2 test; [3]Comparison between controls and all PE group; [4]Comparison between controls and PE-only group; [5]Comparison between controls and PE FGR group. CI: Confidence intervals; NS: Non significant; PE: Pre-eclampsia; FGR: Fetal growth retardation; CagA: Cytotoxin-associated antigen A; VacA: Vacuolating cytotoxin A; HspB: Heat shock protein B; FlagA: Flagellin A; Ure: Ureases.

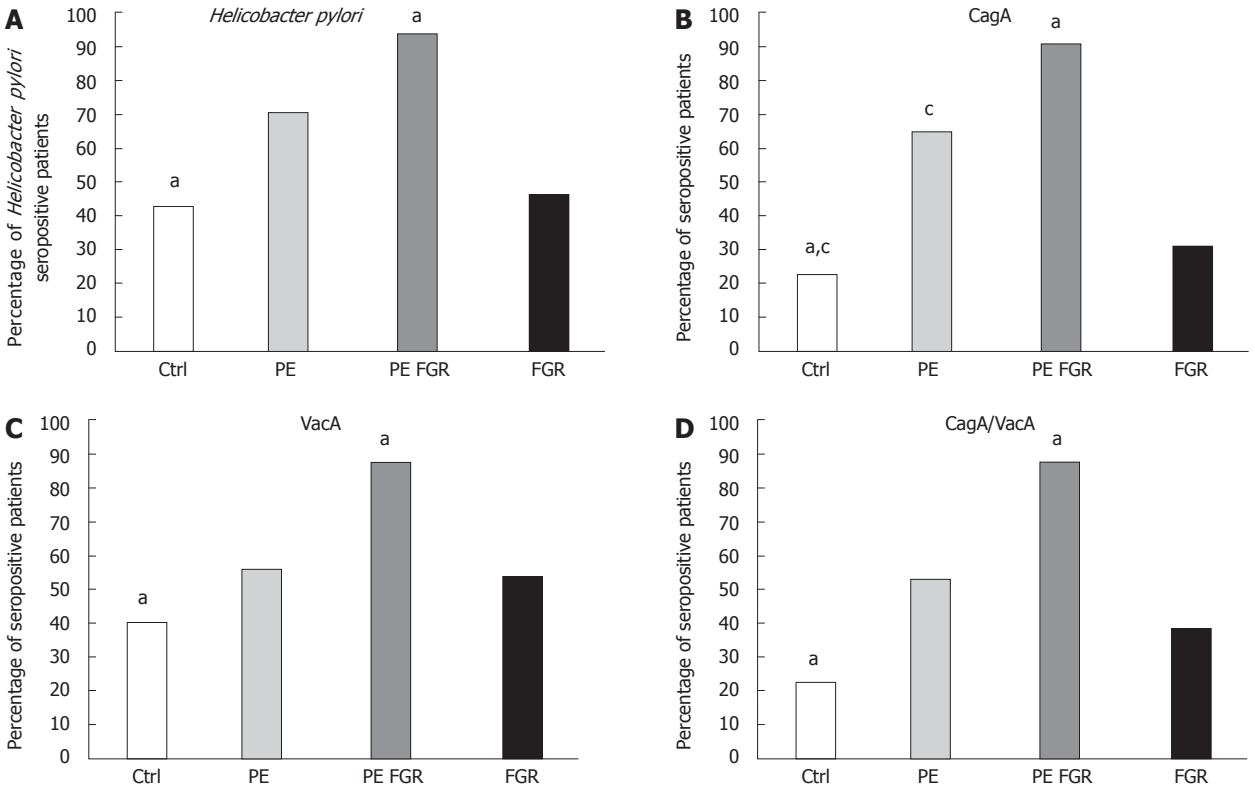

Figure 1 Percentage of *Helicobacter pylori* (A), CagA (B), VacA (C), and CagA/VacA (D) seropositive women in control, PE, PE FGR, and FGR groups. [a]*P* < 0.05 between controls and PE-FGR; [c]*P* < 0.05 between controls and PE without FGR. CagA: Cytotoxin-associated antigen A; VacA: Vacuolating cytotoxin A; PE: Pre-eclampsia; FGR: Fetal growth retardation.

Table 5 Cytotoxin-associated antigen A/vacuolating cytotoxin A dual seropositivity *n* (%)

	Controls (*n* = 49)	All PE (*n* = 49)	PE-only (*n* = 17)	PE FGR (*n* = 32)	FGR-only (*n* = 13)	*P* value[1,2]	Odds ratio[1] (95% CI)
CagA+VacA+	11 (22.4)[3,4]	37 (75.5)[3]	9 (52.9)	28 (87.5)[4]	5 (38.5)	[3]< 0.001	12.10 (3.76-38.91)
						[4]0.001	45.44 (7.79-265.18)
CagA-VacA+	9 (18.4)	0 (0)	0 (0)	0 (0)	1 (7.7)	NS	
CagA+VacA-	0 (0.0)	3 (6.1)	2 (11.8)	1 (3.1)	0 (0.0)	NS	
CagA-VacA-	29 (59.2)[3,4]	9 (18.4)[3]	6 (35.3)	3 (9.4)[4]	7 (53.8)	[3]0.001	0.13 (0.04-0.42)
						[4]< 0.001	0.04 (0.01-0.22)

[1]Adjusted for maternal age at delivery, pre-pregnancy body mass index, parity, and presence of maternal and family risk factors; [2]*P* values were calculated by chi-square test (χ^2); [3]Comparison between controls and all PE group; [4]Comparison between controls and PE FGR group. CI: Confidence intervals; NS: Non significant; CagA: Cytotoxin-associated antigen A; VacA: Vacuolating cytotoxin A; PE: Pre-eclampsia; FGR: Fetal growth retardation.

patients seropositive for UreC (OR 4.02, 95% CI: 1.27-12.8) and UreE (OR 6.29, 95% CI: 1.88-21.04) (Table 4).

Association among CagA/VacA seropositivity and leukocyte blood count, platelet count, and serum amino-transferases values

Considering seropositivities for CagA and/or VacA antigens, we found that the total leukocyte count was significantly decreased in VacA only seropositive patients relative to seronegative, CagA+/VacA- and CagA+/VacA+ patients ($P = 0.003$; $P = 0.014$ and $P = 0.012$, respectively, Table 6). Moreover, the basophiles percentage, but not total count, was significantly increased in CagA/VacA double seropositive compared to seronegative patients ($P = 0.002$, Table 6). No differences among groups were found for the other investigated parameters (Table 6). Analyzing amino-transferases levels after HELLP cases exclusion, ALT was significantly increased in CagA only seropositive patients relative to the other groups ($P = 0.02$; $P = 0.025$; $P = 0.023$, respectively, Table 6), while no differences were found for AST levels (Table 6).

DISCUSSION

In the present study, we reported a direct association between *H. pylori* virulence and the onset of pre-eclampsia complicated by FGR. Moreover, by investigating seropositivity for *H. pylori* virulence factors, we were able to

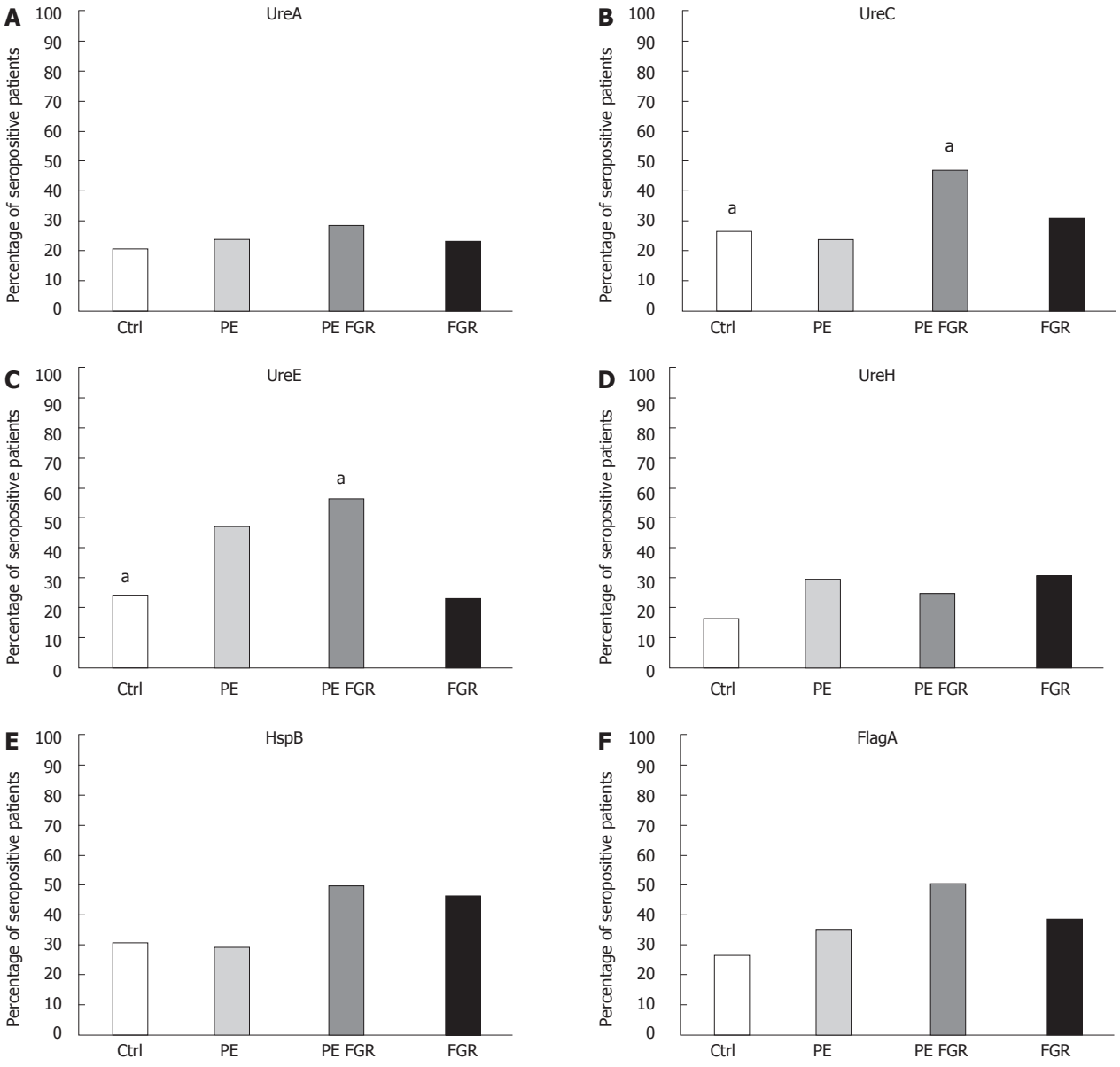

Figure 2 Percentage of Ureases (A-D), HspB (E), and FlagA (F) seropositive women in control, PE, PE FGR, and FGR groups. $^{a}P < 0.05$ *vs* controls. HspB: Heat shock protein B; FlagA: Flagellin A; Ure: Urease; PE: Pre-eclampsia; FGR: Fetal growth retardation.

distinguish pre-eclampsia and FGR without hypertension as different pathologies.

It is accepted that pre-eclamptic pregnancies, complicated or not by FGR, are characterized by severe maternal inflammation[1]. Less is known about "pure" FGR pregnancies, probably because of a biased classification system that considers FGR a secondary disease or a complication of pre-eclampsia. We found elevated maternal leukocytes count, typical sign of inflammation, in all pathological pregnancies relative to controls. However, while leukocytosis in PE patients, as previously reported[29,30], was mainly due to elevated neutrophils levels, a typical marker of bacterial infection[31], in FGR-only mothers, leukocytosis was due to increase in monocytes, eosinophils, and lymphocytes. Moreover, we found significantly higher transaminases levels in the PE group, even after the exclusion of HELLP cases, known to be characterized by elevated hepatic enzymes. The trigger of this exacerbated inflammatory response still remains unknown.

Graham *et al*[21] previously demonstrated a direct association between abnormal total leukocyte count and *H. pylori*-infection in patients with duodenal ulcer disease. They reported a significant fall in total white cell and neutrophils counts in patients successfully treated by *H. pylori* antibiotic therapy[21]. Moreover, they observed higher AST levels in CagA-positive patients, even after antibiotic treatment, thus assuming that AST levels are not directly associated with *H. pylori* infection[21]. Furthermore, we previously reported a correlation between *H. pylori* infec-

Table 6 Hematological values and cytotoxin-associated antigen A/vacuolating cytotoxin A antigens

Variables	Normal values in Italian female population range	CagA-VacA- (n = 45) Median (25th-75th)	CagA-VacA+ (n = 10) Median (25th-75th)	CagA+VacA- (n = 3) Median (25th-75th)	CagA+VacA+ (n = 53) Median (25th-75th)	P value[2]
Total leukocyte count ($1 \times 10^3/\mu L$)	4.00-11.00	12.02 (10.6-13.13)[3]	8.95 (7.75-10.5)[3,5,6]	14.1 (12.4-16.34)[5]	11.27 (9.62-13.64)[6]	[3]0.003; [6]0.012; [5]0.014
Neutrophils ($1 \times 10^3/\mu L$)		10.02 (8.88-11.40)	7.45 (4.87-9.48)	12.75 (11.03-14.48)	8.43 (6.9-10.5)	NS
(%)	45.0-73.0	78.8 (67.05-88.85)	71.9 (68.1-77.3)	83.4 (78.2-88.6)	72.05 (67.4-80)	NS
Lymphocytes ($1 \times 10^3/\mu L$)		1.72 (1.11-2.85)	2.13 (1.68-2.13)	1.61 (0.93-2.28)	2.34 (1.63-3.07)	NS
(%)	19.0-47.0	15.3 (8.9-23.2)	20.6 (17.4-23.5)	10.95 (5.7-16.2)	18.75 (14-23.3)	NS
Monocytes ($1 \times 10^3/\mu L$)		0.57 (0.26-0.89)	0.54 (0.43-0.6)	0.78 (0.68-0.88)	0.6 (0.44-0.84)	NS
(%)	3.0-9.0	4.75 (2.15-6.85)	5.2 (4.9-6)	5.1 (4.8-5.4)	5.81 (4.3-7.4)	NS
Eosinophils ($1 \times 10^3/\mu L$)		0.05 (0.02-0.18)	0.09 (0.03-0.21)	0.06 (0.03-0.08)	0.06 (0.03-0.15)	NS
(%)	0.2-4.4	0.55 (0.1-1.45)	1.2 (0.2-2)	0.4 (0.2-0.6)	0.58 (0.3-1.17)	NS
Basophils ($1 \times 10^3/\mu L$)		0.01 (0-0.02)	0.03 (0.01-0.03)	0.02 (0.02-0.03)	0.03 (0.01-0.04)	NS
(%)	0.1-1.3	0.1 (0-0.2)	0.2 (0.2-0.3)	0.15 (0.1-0.2)	0.2 (0.2-0.3)	0.002
Platelets[1] ($1 \times 10^3/\mu L$)	150-400	222 (169-249)	175 (154-209.5)	214 (210-228)	191 (166-242)	NS
ALT[1] (U/L)	< 34	16 (12.5-26)[4]	11 (9-12)[5]	32 (30-178)[4,5,7]	17 (11.5-24)[7]	[4]0.020; [5]0.025; [7]0.023
AST[1] (U/L)	< 31	18 (15.5-23.5)	13 (12-19)	58 (15-143)	18 (14.5-26)	NS

[1]Hemolysis-elevated liver enzymes-low platelets cases excluded; [2]P values were calculated by non-parametric Kruskal-Wallis H test, with post-hoc analysis by Mann-Whitney U test; [3]Comparison between CagA-VacA- and CagA-VacA+ groups; [4]Comparison between CagA-VacA- and CagA+VacA- groups; [5]Comparison between CagA-VacA+ and CagA+VacA- groups; [6]Comparison between CagA-VacA+ and CagA+VacA+ groups; [7]Comparison between CagA+VacA- and CagA+VacA+ groups. CagA: Cytotoxin-associated antigen A; VacA: Vacuolating cytotoxin A; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; NS: Non significant.

tion and the onset of pre-eclampsia during pregnancy, suggesting that this Gram-negative bacterium could cause or contribute to the etiopathogenesis of pre-eclampsia[7], by inducing the pro-inflammatory state.

In the present study, we further investigated *H. pylori* and pre-eclampsia association by considering the main markers of *H. pylori* virulence and infection persistence, which are useful for understanding the severity and characteristics of the infection.

H. pylori strains carrying the CagA antigen are known to be among the most virulent and are associated with increased inflammation[13]. VacA is a *H. pylori* toxin crucial to promote and maintain bacterial colonization[14]. Importantly, combined seropositivity for both CagA and VacA directly correlates with elevated morbidity[32-34]. We previously reported a strong association between CagA positive *H. pylori* infection and the onset of PE in Italian women[7]. In the present study, we also found that CagA/VacA dual seropositivity is specifically associated with pre-eclampsia and, in particular, with PE complicated by FGR. In contrast, the absence of both anti-CagA and anti-VacA antibodies is associated with a lower risk of PE. Interestingly, the association with CagA-/VacA+ was found only in controls and normotensive women with FGR pregnancies, while CagA+/VacA- patients belong to the PE groups. Our data suggest that the CagA antigen is associated with a more severe pattern, while VacA alone is not sufficient to cause the severe systemic inflammation typical of PE. The highest leukocyte count and ALT level observed in CagA+/VacA- patients further corroborated this hypothesis, while subjects seropositive only for VacA were characterized by the lowest median leukocyte values (Table 6). CagA/VacA dual seropositivity was the most frequent condition in PE complicated by FGR patients (Table 5), and was associated with intermediate leukocyte and ALT values (Table 6). Therefore, we speculate that CagA, with or without VacA, may contribute to the onset of pre-eclampsia, while VacA seropositivity could attenuate CagA virulence. Our results indicate that severe (CagA positive) and persistent (VacA positive) maternal *H. pylori* infections are strongly associated to pre-eclampsia complicated by feto-placental compromise, as indicated by FGR. Therefore, chronic and severe *H. pylori* infections could contribute not only to the exacerbated maternal inflammatory response leading to pre-eclampsia, but also to the abnormal placentation typical of FGR.

Importantly, FGR without PE does not present significant differences relative to physiological controls for either *H. pylori* (Table 3, Figure 1A) and CagA/VacA dual seropositivity (Figure 1D), suggesting that different etiopathogenetic mechanisms lead to "pure" FGR.

Another key *H. pylori* virulence factor is Urease, an enzyme that modifies environmental pH to allow *H. pylori* colonization[35]. Moreover, it helps to activate pro-inflammatory cytokines production[15]. We determined seropositivity for A, E and H urease subunits and for UreC in pre-eclamptic and/or FGR pregnant women relative to controls. In PE women relative to controls, we found significantly higher seropositivity only for the UreE subunit; the carrier of nickel ions and pivotal for proper enzyme activity[36-38]. The rate of seropositivity for UreC, the enzyme necessary for bacterial cell wall formation[39], was significantly higher in PE pregnancies complicated by FGR, as we previously showed for CagA/VacA dual-seropositivity. These data suggest that UreE and UreC contribute to the onset of both PE and PE-FGR.

Even though pre-eclampsia has been extensively investigated, the only effective therapeutic option remains a timed, programmed delivery. Our data clearly demonstrate a direct correlation between severe and persistent *H. pylori* infection and the onset of PE complicated by FGR, opening up attractive perspectives for the design of new preventive and therapeutic interventions for pre-eclampsia.

Although specific combinations of different antibiotics are effective in eradicating *H. pylori*, antibiotics-resistant strains are already emerging, thus decreasing the efficacy of existing therapies[40]. Pharmacogenomics-based treatments seem to increase the cure rates and new therapeutic approaches targeting *H. pylori* virulence factors are required[40]. In the case of pregnancy-related diseases, it would be preferable to prevent the exacerbated inflammation typical of PE, thus avoiding pharmacologic therapies during pregnancy. Recently, several clinical trials and animal studies have focused on generating *H. pylori* recombinant vaccines[41,42]. They demonstrated the possibility of eliciting an immunological response against *H. pylori* in humans, and to eradicate and protect against the infection in mice[43]. Experimental *H. pylori* vaccines have been created using bacterial urease and designed as oral preparations.

In conclusion, our results define pre-eclampsia complicated by FGR and "pure FGR" as different pathologies. Moreover, we demonstrated a direct role for *H. pylori* CagA/VacA positive strains in the etiopathogenesis of PE-FGR. Our data further emphasize the importance of an accurate classification of the multifactorial and multiform pre-eclamptic disease. It is generally accepted that PE is a syndrome that includes several pathologies with different etiopathogenesis but with similar clinical manifestations. For this reason, PE is usually classified on the basis of symptoms severity (moderate or severe) or of symptoms onset (early- or late-onset PE). We strongly believe that, as demonstrated by the present study, pre-eclampsia should also be classified as placental (with feto-placental involvement) or maternal (without feto-placental compromise)[44], both of which may have early or late onset. This classification will lead to a better management of this devastating pregnancy-related disorder. Further studies are required to identify specific *H. pylori*-related therapeutic targets.

COMMENTS

Background

Pre-eclampsia (PE), a severe hypertensive pregnancy-related syndrome that affects 5%-8% of women worldwide, represents the main cause of feto-maternal mortality and morbidity. Despite being the object of intense investigation, the etiopathogenesis of PE is still poorly understood, and no effective therapeutic interventions are available in clinical practice.

Research frontiers

Several lines of evidence suggest that maternal sub-clinical infections could play a pivotal role in the onset of PE. *Helicobacter pylori* (*H. pylori*) could directly cause or intensify the generalized inflammation and endothelial dysfunction typical of this syndrome.

Innovations and breakthroughs

The data represent a major advance in the understanding of PE etiopathogenesis and add pivotal information for an accurate classification of this multifactorial and multiform syndrome. In fact, the authors clearly demonstrated a direct correlation between severe and persistent *H. pylori* infection and the onset of PE complicated by fetal growth retardation (FGR).

Applications

The findings open up new, attractive perspectives regarding the design of effective preventive and therapeutic interventions for pre-eclampsia associated with *H. pylori* infection.

Terminology

FGR is defined as failure of the fetus to achieve its genetically determined growth potential and is commonly considered a severe complication of PE.

Peer review

The work is a contribution to the understanding of *H. pylori*'s pathogenic role in PE, associated or not with FGR. The association between maternal infection and PE has been evaluated by several researchers and is a good field to study the etiopathogenesis of this critical clinical condition. Authors confirmed that persistent and virulent *H. pylori* infections cause or contribute to PE complicated by FGR.

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BRIEF ARTICLE

Infliximab in pediatric inflammatory bowel disease rapidly decreases fecal calprotectin levels

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Abstract

AIM: To study the response to infliximab in pediatric inflammatory bowel disease (IBD), as reflected in fecal calprotectin levels.

METHODS: Thirty-six pediatric patients with IBD [23 Crohn's disease (CD), 13 ulcerative colitis (UC); median age 14 years] were treated with infliximab. Fecal calprotectin was measured at baseline, and 2 and 6 wk after therapy, and compared to blood inflammatory markers. Maintenance medication was unaltered until the third infusion but glucocorticoids were tapered off if the patient was doing well.

RESULTS: At introduction of infliximab, median fecal calprotectin level was 1150 μg/g (range 54-6032 μg/g). By week 2, the fecal calprotectin level had declined to a median 261 μg/g ($P < 0.001$). In 37% of the patients, fecal calprotectin was normal (< 100 μg/g) at 2 wk. By week 6, there was no additional improvement in the fecal calprotectin level (median 345 μg/g). In 22% of the patients, fecal calprotectin levels increased by week 6 to pretreatment levels or above, suggesting no response (or a loss of early response). Thus, in CD, the proportion of non-responsive patients by week 6 seemed lower, because only 9% showed no improvement in their fecal calprotectin level when compared to the respective figure of 46% of the UC patients ($P < 0.05$).

CONCLUSION: When treated with infliximab, fecal calprotectin levels reflecting intestinal inflammation normalized rapidly in one third of pediatric patients suggesting complete mucosal healing.

Key words: Crohn's disease; Ulcerative colitis; Surrogate markers; Pediatrics; Monoclonal antibodies; Infliximab

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INTRODUCTION

The recent development of easily applicable fecal surrogate markers for intestinal inflammation has provided

new means for objective assessment of disease activity and treatment response in chronic inflammatory bowel disease (IBD), a disease becoming more prevalent among children[1]. This is especially important in pediatric patients with limited possibility for follow-up endoscopy due to invasiveness. The fecal levels of neutrophil-derived markers, such as fecal calprotectin or lactoferrin, reflect the mucosal influx of inflammatory cells in the intestine, thus associating with the presence of active inflammation. In IBD, fecal calprotectin levels relate to the findings in endoscopy but also with the grade of histological inflammation[1-4]. When compared to clinical scores and serum inflammatory markers, fecal calprotectin is the most accurate tool to detect the presence of active mucosal inflammation in the colon[4-6]. The negative predictive value for the presence of active inflammation is high (87%)[4]. In children, it has been shown that the level of fecal calprotectin[2,3,7,8] or lactoferrin[6,9] may guide the need for endoscopy.

The data on fecal markers during therapy of IBD are sparse. We showed recently that during glucocorticoid therapy in pediatric patients, fecal calprotectin levels rarely declined below the limit of a raised value, suggesting ongoing mucosal inflammation. However, in those clinically responding to therapy, fecal calprotectin values fell markedly during the first month of therapy[10]. In children presenting with clinically quiescent IBD, only one third of the patients have fecal calprotectin levels below the upper normal limit, whereas the others have raised values, although not reporting subjective symptoms[11]. In adults, fecal calprotectin values are associated with mucosal healing in Crohn's disease (CD) patients who respond to therapy with tumor necrosis factor (TNF)-α antagonists or other IBD medication[12,13]. In a pilot study by Buderus *et al*[14], the levels of fecal lactoferrin were measured in five children on infliximab therapy, who showed a decline after the first infusion in each case. The pattern of fecal calprotectin levels during introduction of TNF-α antagonist therapy in children has not yet been described.

In pediatric patients, TNF-α antagonists have emerged for therapy of severe IBD that does not respond to conventional treatment[15-17]. Fecal calprotectin provides a non-invasive means to assess the presence of intestinal inflammation, therefore, we conducted a prospective study in pediatric patients treated with TNF-α antagonist infliximab. Our aim was to study the pattern of fecal calprotectin concentrations during the early phase of therapy.

MATERIALS AND METHODS

Study population

We prospectively studied 36 children (median age 14 years, range 5.6-17.6 years; 20 boys 20, 16 girls) diagnosed with IBD according to the Lennard-Jones criteria[18], and consecutively introduced to therapy with infliximab. In two cases, the diagnosis of CD was based on extensive aphthous ulceration visualized by wireless capsule endoscopy. All patients had moderate to severe disease that did not respond to treatment with 5-aminosalicylic acid (5-ASA), immunosuppressants or glucocorticoids. In four cases, infliximab was introduced shortly after a diagnosis of extensive small bowel disease. In three patients, the indication for anti-TNF-α agent was fistulating disease, and in all the others, poor response to maintenance medication or steroid dependency. The study group comprised 23 pediatric patients with CD, and 13 with ulcerative colitis (UC). The background data, disease distribution, and medication of the patients are shown in Table 1. Fourteen patients underwent ileocolonoscopy, seven underwent wireless capsule endoscopy, and seven magnetic resonance imaging enterography within 1 m prior to the introduction of infliximab therapy, confirming active disease.

Table 1 Background data of 36 pediatric patients with inflammatory bowel disease treated with infliximab

Variable	Result
Age median (range)	14 (5.6-17) yr
Sex	20 boys, 16 girls
Diagnosis	
CD	23
Ileitis	8
Ileocolitis	8
Colitis	7
UC	13
Left-sided colitis	4
Pancolitis	9
Maintenance medication at baseline	
5-ASA	10
5-ASA + azathioprine/6-MP	12
Azathioprine/6-MP	7
None	7
Prednisolone/budesonide at baseline	19
Disease duration (median, range)	2.1 (0.4-7.6) yr

CD: Crohn's disease; UC: Ulcerative colitis; 5-ASA: 5-aminosalicylic acid; 6-MP: 6-mercaptopurine.

TNF-α antagonist infliximab (Remicade®) was scheduled at 5 mg/kg at weeks 0, 2 and 6. All infusions were administered at the Hospital for Children and Adolescents, Helsinki University, Finland during February 2008 to December 2010. The maintenance medication was unaltered until week 6, but if the patient improved clinically, glucocorticoids were tapered off. At each visit, the patients provided a stool sample for fecal calprotectin measurement and a blood sample for measurement of inflammatory marker erythrocyte sedimentation rate (ESR), and hemoglobin. Fecal calprotectin was measured in the routine clinical laboratory by a quantitative enzyme immunoassay (PhiCal Test, Calpro AS, Oslo, Norway; NovaTec Immunodiagnostica, Dietzenbach, GmBH, Germany) and the values quoted as normal were < 100 μg/g stool[10,19]. The clinical activity of the disease was assessed by physicians global assessment (PGA) score from 1 to 3[20].

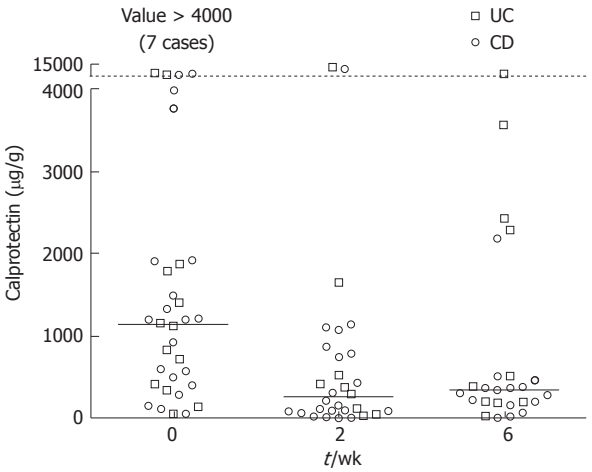

Figure 1 Fecal calprotectin levels at baseline, and 2 and 6 wk after introduction of infliximab therapy in children with Crohn's disease or ulcerative colitis. The decline in levels between baseline and week 2 was statistically significant ($P < 0.001$). CD: Crohn's disease; UC: Ulcerative colitis.

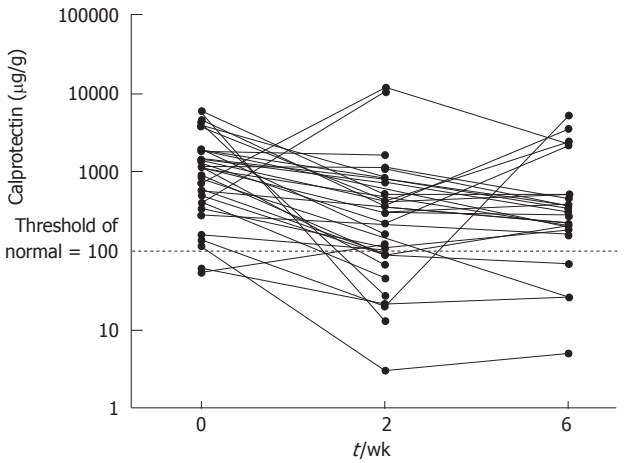

Figure 2 Fecal calprotectin levels at baseline, and 2 and 6 wk after introduction of infliximab therapy showing the individual variation in children with inflammatory bowel disease.

Ethical considerations

The ethics committee of Helsinki University Central Hospital approved the study protocol. The families attending the study signed an informed consent form.

Statistical analysis

Spearman non-parametric correlation test, Kruskal-Wallis test, Mann-Whitney *U* test, and Fisher's exact test were used. The level of statistical significance was $P < 0.05$. The values are presented as median and range.

RESULTS

Fecal calprotectin was high at the introduction of infliximab therapy, with a median value of 1150 μg/g (range: 54-6032 μg/g). Two patients had fecal calprotectin < 100 μg/g (reference limit for a raised value), and their indication for treatment was steroid-dependent colitis. By week 2, the median level of fecal calprotectin level had declined to 261 μg/g (Figure 1; $P < 0.001$, Mann-Whitney *U* test). In 11 of 30 (37%) patients, fecal calprotectin was below the reference limit (100 μg/g) by week 2. By week 6, there was no additional improvement in the median fecal calprotectin level (345 μg/g, range: 5-5253 μg/g, Figure 1). The individual variation of fecal calprotectin levels is shown in Figure 2.

Disease extension or diagnosis did not relate to fecal calprotectin levels or to treatment response (data not shown). Fecal calprotectin decreased in 21 of 22 (95%) of the CD patients, with a raised value during the introduction phase, but in one case, the response was temporary. By week 6, there was no response in two cases when compared to baseline. One patient with CD presented with low fecal calprotectin levels throughout the study period. In this particular case, the indication for treatment was steroid dependency and steroids were tapered off during induction. Thus, the effect of infliximab therapy on fecal calprotectin could not be assessed, and she was not included in the analysis. Of the UC patients with increased fecal calprotectin at baseline, the level decreased in 10 of 12 (83%) patients, but increased to pretreatment levels or above by week 6 in three of 10 (30%) children. Of the two children with no initial decline in fecal calprotectin level, there was no clinical response and the level stayed constantly high (> 1600 μg/g) or increased more than 10-fold within 6 wk. The child with steroid-dependent UC and low fecal calprotectin at the start of this therapy (high disease activity confirmed in colonoscopy 1.5 mo earlier) showed mild elevation in the level (up to 120 μg/g). Thus, by week 6, fecal calprotectin level suggested a response in 20 of 22 (91%) of the CD patients and in seven of 13 (54%) of the UC patients ($P < 0.05$, Fisher's exact two-tailed test), corresponding to a figure of 22% of non-responsive patients in total.

Blood inflammatory marker ESR decreased from a median value of 20 mm/h (range: 2-46 mm/h) at baseline to 9 mm/h (range: 2-34 mm/h, $P < 0.05$, Mann-Whitney *U* test) at 2 wk. At baseline, 19 of 31 patients (61%) had elevated ESR. By week 2, the respective figure was 28% (9/32). Median PGA score was 2 at baseline (range: 1-3), and by week 2 and 6, the score was 1 (range: 1-3, $P < 0.001$); by week 6, the majority of patients (33/36) presented with a score of 1. For hemoglobin levels, there was no significant increase in the median values [118 g/L (range: 95-152 g/L) at baseline and 124 g/L at 2 wk (range: 80-147 g/L)]. Glucocorticoids were tapered off in 10/19 patients during the induction phase.

DISCUSSION

Therapy with TNF-α antagonists has emerged in pediatric patients suffering from moderate to severe CD[21,22], but recently, a therapeutic response has also been reported in severe UC[16,23]. These therapies are effective but at present have high costs and carry a risk for the development of severe adverse effects, which possibly

hampers their clinical use[24,25]. Thus, it is of key importance to target the therapy on those who show a positive response and to discontinue administration of TNF-α antagonists if the patient is a non-responder[25]. In keeping with this, surrogate markers for the presence of intestinal inflammation such as fecal calprotectin[13,26] are promising and non-invasive means for the assessment of disease activity in IBD. There have only been a few studies on fecal calprotectin related to therapeutic response in IBD. Previously, we have assessed the pattern of fecal calprotectin in acute pediatric IBD from the start of glucocorticoid therapy until their discontinuation[10], and in adults during the first 12 wk of TNF-α antagonist therapy[12]. Here, we showed the pattern of fecal calprotectin in pediatric IBD during the induction phase of TNF-α antagonist therapy, demonstrating a rapid decline in fecal calprotectin levels within the first weeks of induction in the majority of pediatric IBD patients, suggesting an early response.

By week 2 after introduction of infliximab, the median level of fecal calprotectin had declined by 77% from baseline. Expectedly, this rapid decrease in fecal calprotectin in children paralleled the pattern seen in adults after introduction of TNF-α antagonist treatment. In a previous study in adults, endoscopy confirmed remission in 30% of CD patients when assessed at 3 mo[12]. In the present study, in one third of the children, the fecal calprotectin levels had declined to normal - suggesting remission - by week 2 after the start of infliximab therapy. Unexpectedly, there were only a few cases that showed normalization of fecal calprotectin by week 6, thus, the 2-wk result equaled the proportion of children with suggested mucosal healing - the target of IBD therapy[27] - and remission during the induction phase. The finding of an excellent therapeutic response in one third of the patients is comparable to our previous findings in children with acute IBD treated with glucocorticoids (showing a normalization of fecal calprotectin in 27% of patients[10]), and in adults treated with TNF-α antagonist therapy[12] (see above). In two thirds of the patients, fecal calprotectin did not normalize, suggesting incomplete mucosal healing during the induction phase of infliximab therapy. It is important to note that the fecal calprotectin level that is considered as a satisfactory therapeutic response remains undecided. Furthermore, the long-term treatment outcome in pediatric patients related to fecal calprotectin levels warrants further studies. In adult patients, mucosal healing predicts a better long-term outcome[28].

Although primary response to TNF-α antagonist therapy is excellent in children, covering 80%-90% of patients with CD, the therapeutic response according to clinical disease activity may deteriorate during the first year of therapy in a considerable proportion of children. It has been estimated that 34%-49% of initial responders need dose escalation or more intense therapy during the first year of infliximab therapy[15,22]. For infliximab therapy in adults, rates of dose intensification ranging from 31% to 36% at 12 mo are comparable to those in pediatric patients[29,30]. Here, the primary response was possibly lost already during the introduction phase in > 10% of the patients, as reflected in the fecal calprotectin levels. In these particular children, PGA did not decrease either. However, clinical activity indices have less correlation to the presence of mucosal inflammation than fecal neutrophil biomarkers, as shown in adult CD patients[31]. Thus, reliance solely on clinical assessment is insufficient, and constantly high fecal calprotectin concentration during therapy warrants endoscopic evaluation also in children. It should be noted that increased fecal calprotectin level does not discriminate between disease relapse and intestinal infection[32].

Blood hemoglobin levels did not significantly alter during the induction phase, but the median ESR decreased during the induction phase by week 2. However, 39% of the patients had normal ESR at baseline, and in these patients, ESR is not applicable for assessment of therapeutic response. In children with IBD, serum C-reactive protein (CRP) is seldom increased, and disappointingly, the measurement of high-sensitivity CRP does not bring additional benefit for the assessment of disease activity[33]. Thus, CRP levels were not measured systematically in the present study. It has also been shown in adult IBD that CRP is a poor marker in mild to moderate disease[34], and performance of fecal calprotectin is significantly better[6].

As in many pediatric studies, one of the major limitations of the present study was the small size of the study group. The majority of the patients had CD but as there were only 13 cases of UC, comparisons related to diagnosis of IBD should be interpreted with caution. Most patients with CD presented with ileocolitis, and the numbers of patients with terminal ileal disease or CD colitis were too small to allow proper comparisons related to therapeutic response to infliximab therapy.

In conclusion, fecal surrogate markers may provide objective and non-invasive means to determine the response to infliximab in individual patients early in treatment. Also, fecal calprotectin is more reliable than clinical activity indices or blood-borne markers of inflammation. It may be possible to identify responding patients by a rapid drop in fecal calprotectin levels, which can be seen already at week 2. By week 6, little improvement is evident and some patients even appear to lose their therapeutic response. Based on our results, fecal calprotectin is a promising marker for evaluating patient response to TNF-α antagonist therapy and may offer a tool for identifying responding patients at an early stage, for more efficient targeting of treatment.

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COMMENTS

Background

In pediatric patients, tumor necrosis factor (TNF)-α antagonists have emerged for therapy of severe inflammatory bowel disease (IBD), in patients who do not respond to conventional treatment. In children, endoscopy is an invasive procedure, which limits its use in follow-up. Fecal calprotectin is a surrogate marker for the presence of intestinal inflammation and thus provides a non-invasive means to assess disease activity in children.

Research frontiers

The recent development of easily applicable fecal surrogate markers for intestinal inflammation has provided new means for objective assessment of disease activity and treatment response in chronic IBD, a disease becoming more prevalent among children. When compared to clinical scores and serum inflammatory markers, fecal calprotectin is the most accurate tool to detect the presence of active mucosal inflammation in the intestine, and it is easily applicable to pediatric clinical practice.

Innovations and breakthroughs

This is believed to be the first pediatric study to follow fecal calprotectin levels during the induction phase of therapy with TNF-α antagonist agent infliximab. The study showed that, in one third of pediatric patients, fecal calprotectin level normalized by week 2. However, in two thirds of the patients, fecal calprotectin levels stayed elevated by week 6, suggesting incomplete mucosal healing.

Applications

Based on the results, fecal calprotectin is a promising marker for objective evaluation of patient response to TNF-α antagonist therapy and may offer a tool for identifying responding patients at an early stage, for more efficient targeting of treatment. The long-term treatment outcome in pediatric patients related to fecal calprotectin levels after induction therapy warrants further study.

Terminology

IBD consists of Crohn's disease, ulcerative colitis and indeterminate colitis, and is a chronic illness that affects the intestines, with a partly TNF-α-driven inflammation that is effectively abated by TNF-α antagonists such as infliximab. Fecal calprotectin is a neutrophil-derived marker of inflammation that is present in the stools, and is a reliable surrogate for endoscopic evaluation of disease activity.

Peer review

The authors prospectively evaluated the therapeutic response in pediatric IBD patients during introduction to infliximab reflected in fecal calprotectin levels. It is a relevant study and the paper is well presented.

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BRIEF ARTICLE

Five methods for detection of *Helicobacter pylori* in the Turkish population

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Abstract

AIM: To compare culture analysis, *Helicobacter pylori* (*H. pylori*) stool antigen (HpSA) test, polymerase chain reaction (PCR) and fluorescence *in situ* hybridization (FISH) for *H. pylori* detection.

METHODS: One hundred and thirty-two consecutive adult dyspeptic patients receiving diagnostic endoscopy at the department of gastroenterology were enrolled in this study. Culture and histological examination were performed on biopsy specimens. PCR and FISH tests were applied to histopathological samples. Stool samples that were simultaneously collected were tested for the *H. pylori* antigen using the HpSA test and bacterial DNA using stool PCR.

RESULTS: *H. pylori* was positively identified by histological examination in 85/132 (64.4%) of the patients, while positive samples were found in 56 (42.4%), 64 (48.5%), 98 (74.2%), 28 (21.2%) and 81 (61.4%) of the patients by culture, HpSA, PCR, stool PCR and FISH methods, respectively. The results of the culture, biopsy PCR, HpSA and FISH tests, with the exception of the stool PCR, were found to correlate with the histological examination as a gold standard.

CONCLUSION: The HpSA test is a rapid, simple, and noninvasive test for monitoring therapy. FISH is an accurate, rapid, cost-effective, and easy-to-use test for *H. pylori* detection.

Key words: *Helicobacter pylori*; Histology; Polymerase chain reaction; *Helicobacter pylori* stool antigen; Fluorescence *in situ* hybridization

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INTRODUCTION

In 1984, Marshall and Warren[1] reported the discovery of a bacterium, which was subsequently named *Helicobacter pylori* (*H. pylori*)[2], whose habitat was the human gastric mucosa. This bacterium has been shown to play a role in gastritis, peptic ulcer disease, and gastric malignancies[3-5]. Colonization of the human gastric mucosa induces chronic gastritis and peptic ulcer disease[3,4]. In addition,

H. pylori plays a role in the etiology of gastric cancer and cancer of the mucosa-associated lymphoid tissue[5].

The accurate detection of *H. pylori* is essential for the management of patients and for the eradication of the bacterium following treatment. Since the discovery of *H. pylori*, several diagnostic methods have become available for determining the presence of *H. pylori* infection. These tests can be assessed by invasive and noninvasive methods[6]. Assessment of *H. pylori* infection is based on noninvasive tests, such as serological methods, C urea breath test, and bacterial DNA sequences or bacterial antigen detection in stool by the *H. pylori* stool antigen (HpSA) test[7]. Under many circumstances, noninvasive testing is preferred. These tests are attractive because of their simplicity and the ability to provide test results within a few minutes after administration, in a physician's office. In contrast, the direct detection and culturing of *H. pylori* for the diagnosis of infection requires gastric biopsy specimens obtained from invasive gastroduodenoscopy[5]. Culture methods require an incubation period of at least 4-7 d. However, it is important to note that *H. pylori* is a fastidious microorganism and is affected by environmental conditions[8,9]. The presence of *H. pylori* or resistance to antimicrobials can be investigated on gastric tissue samples with molecular methods, such as polymerase chain reaction (PCR) and fluorescence *in situ* hybridization (FISH).

The aim of this study was to compare culture analysis, HpSA test, PCR and FISH to histological examination for the detection of *H. pylori*.

MATERIALS AND METHODS

Clinical samples

One hundred and thirty-two consecutive adult dyspeptic patients receiving diagnostic endoscopy at the department of gastroenterology were enrolled in this study. Written informed consent was obtained from all of the patients before endoscopy, and sample collection and approval by the Local Ethical Committee was taken prior to initiation of the project. Patients who underwent partial or complete gastrectomy, those with prior *H. pylori* eradication therapy, or those who were treated with any antibiotics, colloidal bismuth compounds, proton pump inhibitors, or H_2 receptor blockers within the past 4 wk were excluded from the study.

Endoscopy and biopsy sampling

Endoscopy was performed with a PentaxFG-29W (Pentax, Germany) on patients after an overnight fast. Four gastric biopsies (two from the antrum and two from the corpus) were taken from each patient.

Culture

Two gastric biopsy specimens, one from the antrum and one from the corpus, were obtained and placed in Stuart's transport medium. Cooled samples were transported to the laboratory of the Department of Microbiology within 1-2 h after procurement, as previously described[10]. Specimens were inoculated onto brain-heart infusion agar supplemented with sheep blood (10%), vancomycin (10 mg/L), trimethoprim lactate (5 mg/L), cefsoludin (5 mg/L), and amphotericin (5 mg/L). The plates were microaerobically incubated using CampyGen (Oxoid, United Kingdom) at 37 ℃ for up to 7 d. Positive cultures were identified by colony formation and Gram stain morphology as well as positive catalase, oxidase, and urease tests.

Histology

Two gastric biopsy specimens, one from the antrum and one from the corpus, were fixed in 10% formalin in separate containers and were sent to the Pathology Laboratory. Samples were embedded in paraffin wax, cut at 5 μm thickness, and stained with modified giemsa and hematoxylin and eosin. Histological evaluation of the samples for *H. pylori* was performed according to the Modified Sydney system[11]. The pathologist was unaware of the patients' clinical conditions and other test results.

HpSA

Stool samples were tested for *H. pylori* antigen by the monoclonal antigen FemtoLab *H. pylori* Cnx kits (Connex GmbH, Martinsried, Germany) using the manufacturer's protocol. Approximately 0.1 g of stool sample was added to vials that contained 1 mL of sample diluent and then emulsified by vortexing for 15 s. The tip of the vial was snapped off and 50 μL sample and 50 μL conjugate were added to the test well. The strip was rinsed after incubation for exactly (60 ± 5) min at ambient temperature. After washing, 100 μL substrate was added and then incubated for 10 min. Finally, the stop solution was added and the samples were analyzed on a spectrophotometer at a wavelength of 450 nm.

PCR

Gastric biopsies from all of the study subjects were stored at temperatures at or below -70 ℃ until use. Each biopsy was digested with tissue extraction buffer at 55 ℃ for 3 h. Then, 200 μL phenol was added to the tissue lysate to extract genomic DNA. *H. pylori* genomic DNA from stool samples was extracted according to Gramley *et al*[12]. Genomic DNA was subsequently quantified by PCR with 16S rRNA. Amplified fragments were separated on a 1% agarose gel and visualized under ultraviolet light.

Fluorescence in situ hybridization

Formalin-fixed paraffin-embedded gastric biopsies were sectioned and dehydrated. The sections were then air-dried and hybridized using the commercially available test system Seafast® *H. pylori* Combi Kit (Izinta, Hungary) according to the manufacturer's instructions. The oligonucleotide probe Hpy-1, which targets a specific sequence of 16S rRNA from *H. pylori*, was hybridized to the sections. Evaluation was performed with a fluorescent microscope equipped with a filter for green fluores-

Table 1 Statistical analysis according to standard test

Method	Sensitivity	Specificity	PPV	NPV	OR	RR
Culture	0.6118	0.9149	0.9286	0.4342	16.94	2.14
HpSA	0.7222	0.6667	0.8125	0.4545	5.20	1.79
Biopsy PCR	0.8824	0.5106	0.7653	0.2941	7.83	2.60
Stool PCR	0.2118	0.7872	0.6429	0.6442	0.99	1.00
FISH	0.9294	0.9574	0.9753	0.1176	296.25	8.29

PPV: **Positive predictive value; NPV: Negative predictive value; OR:** Odds ratio; **RR: Relative risk; HpSA:** *Helicobacter pylori* stool antigen; **PCR:** Polymerase chain reaction; **FISH: Fluorescence** *in situ* hybridization.

Table 2 Test results compared to standard test

Method	False positive	False negative	*r*	*P* value
Culture	4	33	0.510[1]	< 0.001
HpSA	12	20	0.276[1]	< 0.002
Biopsy PCR	23	10	0.430[1]	< 0.001
Stool PCR	10	67	0.001	> 0.05
FISH	2	6	0.872[1]	< 0.001

[1]Correlation is significant at the 0.01 level. HpSA: *Helicobacter pylori* stool antigen; **PCR: Polymerase chain reaction; FISH: Fluorescence** *in situ* hybridization.

cence (Nikon Eclipse 600, Japan).

Statistical analysis

The χ^2 and Pearson correlation analysis were conducted and the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), *P* value, *r* value, odds ratio (OR) and relative risk (RR) were calculated using standard formulas for data using SPSS v. 10.0 (IBM, United States).

RESULTS

H. pylori was identified by histological examination in 85/132 (64.4%) of the patients, while 47/132 (35.6%) of the patients were classified as *H. pylori* negative. Furthermore, positive results were obtained in 56 (42.4%), 64 (48.5%), 98 (74.2%), 28 (21.2%) and 81 (61.4%) of patients by the culture method, HpSA analysis, PCR, stool PCR and FISH, respectively. Histological examination results were evaluated by the gold standard, and specificity, sensitivity, PPV and NPV were calculated for each test (Table 1). A high number of false-positive results was observed in the biopsy PCR (23/98; 23.4%). However, a higher rate of false-negative results was obtained with the culture method (33/76; 43.4%). The culture method, biopsy PCR, HpSA and FISH tests were found to correlate with the Pearson correlation analysis. Similarly, these tests were statistically comparable to the histological examination based on the *P* value with the χ^2 test. In contrast, the stool PCR test did not correlate or have a significant *P* value. These data are summarized in Table 2.

DISCUSSION

There are currently several different diagnostic tests that exist for detecting *H. pylori* infection. Each test has its own merits and demerits in terms of indication, sensitivity, specificity, cost and time. Several studies have examined the diagnostic performance of invasive and non-invasive methods[6,7,9,12,13]. However, these studies were biased or demonstrated a lack of agreement[13]. One possible reason for the discrepancies in diagnostic performance might be due to the selection of various reference methods. Currently, there is no established method to provide a definitive or standard diagnosis of *H. pylori* infection. The selection of tests or the use of a combination of tests without identifying any one specific test as a reference standard can introduce bias[14].

One limit of histological detection of *H. pylori* in gastric biopsy specimens is interobserver variability in assessment[15,16]. A meta-analysis has reported that histological examination results have an approximate sensitivity of 0.70 and specificity of 0.90[17]. This may be due to the discrepancies in the evaluation of features of *H. pylori* or the observations of the pathologist, because pathology results are based on subjective interpretation of different features and classification. Various studies on the reproducibility of histopathological data have reached a similar conclusion. However, the histological examination of the gastric biopsy specimen is accepted as the gold standard for the diagnosis of *H. pylori*[18]. In this study, histological examination resulted in 64.4% positivity for *H. pylori*, which showed a good correlation with the positive detection rates of other methods, with the exception of stool PCR.

Culturing biopsy specimens cannot be routinely used because it is time consuming and very difficult to maintain strict anaerobic conditions. However, bacterial cultures can surely provide specific results and informative data. Gisbert and Abraria have reported three studies with culture sensitivity of 0.45 and specificity of 0.98 in 2006[17]. Similarly, we found that the culture sensitivity and specificity was 0.61 and 0.91, respectively. In addition, the statistical analysis showed a PPV of 0.93, NPV of 0.43, OR of 16.94, and RR of 2.14 compared to histological examination.

The HpSA test is available and recommended in the Maastricht 2-2000 Consensus Report[19] for the pretreatment diagnosis of *H. pylori* infection and confirmation of a *H. pylori* cure following treatment. In a Japanese study, the HpSA test had a reported sensitivity of 93.9% and specificity of 95.7%, compared to a diagnosis of infection based on histological examination[20]. However, Blanco *et al*[21] have observed that another stool antigen test showed a low sensitivity (75%-79%), in patients with *H. pylori* infection who were tested after erradication therapy. We studied the accuracy of the HpSA test in the Turkish population. The HpSA test had a sensitivity of 0.72, specificity of 0.67, accuracy of 0.77, PPV of 0.81, OR of 5.20 and RR of 1.79. Thus, the HpSA test results had a low but acceptable correlation with the histological examination.

It has been reported that the FISH method is an accurate, inexpensive, rapid test for the detection of *H.*

pylori in paraffin-embedded gastric biopsy samples, with a high sensitivity and specificity[22-24]. In addition, it can be applied to fresh gastric tissue samples and *H. pylori* isolates from culture[25]. In this study, the FISH method had a strong correlation with the histological examination and exhibited a sensitivity of 0.93, specificity of 0.96, accuracy of 0.94, PPV of 0.98, OR of 296.25 and RR of 8.29. Furthermore, the FISH method may be a very useful *H. pylori* diagnostic tool in microbiology in the future.

In gastric tissue, the presence of *H. pylori* and resistance genes can be investigated by PCR. It has a high sensitivity and specificity, and can be used as a follow-up assessment after therapy[26,27]. In this study, biopsy PCR studies had a sensitivity of 0.88, specificity of 0.51, accuracy of 0.75, PPV of 0.77, OR of 7.83 and RR of 2.60. Moreover, we found that the specificity value was particularly low for the biopsy PCR results. However, there was a discrepancy between our study and previous reports in terms of the specificity of *H. pylori* detection[28,29]. Lunet *et al*[28] have reported a difference in *H. pylori* positivity by histology *vs* PCR from different populations, in Mozambique and Portugal of 63.7% *vs* 93.1% and 95.3% *vs* 98.1%, respectively. Two possibilities could explain this conflicting result. First, a low density of *H. pylori* colonization may explain the histological results. Alternatively, the PCR results may be reliable because of the use of a specific primer for the particular population.

The stool PCR results had a very low sensitivity and OR (0.21 and 0.99) and had no significant correlation with the histological examination. Previous studies and our data clearly indicate that there is no clinical value in the determination of *H. pylori* in human feces by PCR because of insufficient sensitivity, specificity, and accuracy[30].

There are a variety of tests available for the diagnosis of *H. pylori* infection. Therefore, it is important that laboratories choose the test or tests that are appropriate for the conditions of the laboratories, patient numbers, costs, and account for the need to prepare their own diagnostic algorithms.

In conclusion, the culture, biopsy PCR, HpSA test, and FISH methods for the detection of *H. pylori* in this study, with the exception of stool PCR, were found to correlate with histological examination as a gold standard. In addition, there was a conflicting result on biopsy PCR data when compared to histological examination. The HpSA test is a rapid, simple, and noninvasive test with acceptable results that can be used for monitoring therapy. The FISH method is an accurate, rapid, cost-effective and easy-to-use test for the detection of *H. pylori*, and also allows for the simultaneous determination of antibiotic resistance in the same gastric tissue. Therefore, histopathological examination as a gold standard and the FISH test may be the preferred methods to use together for the precise detection of *H. pylori*.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) plays a role in gastritis, peptic ulcer disease and also gastric malignancies such as gastric cancer and cancer of the mucosa-associated lymphoid tissue. The accurate detection of *H. pylori* is essential for the management of patients and eradication of the bacteria following treatment.

Research frontiers

Since the discovery of *H. pylori*, several diagnostic methods have been become available for determining the presence of *H. pylori* infection. However, there is no established method to provide a definitive or standard diagnosis of *H. pylori* infection.

Innovations and breakthroughs

The fluorescence *in situ* hybridization (FISH) test is an accurate, rapid, inexpensive and easy-to-use method for the detection of *H. pylori*, and allows determination of antibiotic resistance in the same gastric tissue simultaneously. In this study, FISH correlated well with histological examination. Therefore, histological examination and the FISH test may be preferred together for the precise detection of *H. pylori*.

Applications

This study suggests that, laboratories choose the test or tests that are appropriate for their own conditions, patient numbers and costs, and have to prepare their own diagnostic algorithms.

Terminology

For the detection of *H. pylori*, culture, *H. pylori* stool antigen test, polymerase chain reaction and FISH were used with histological examination.

Peer review

This was an interesting study, although a few problems need to be resolved before publication. The most important point is the reliability of their gold standard. The reasons for the false-positive and false-negative results of each test should be discussed further.

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BRIEF ARTICLE

Spectrum of final pathological diagnosis of gastric adenoma after endoscopic resection

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Abstract

AIM: To investigate how many discrepancies occur in patients before and after endoscopic treatment of referred adenoma and the reason for these results.

METHODS: We retrospectively reviewed data from 554 cases of 534 patients who were referred from primary care centres for adenoma treatment and treated for endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD) at Chungnam National University Hospital, from July 2006 to June 2009. Re-endoscopy was examined in 142 cases and biopsy was performed in 108 cases prior to treatment. Three endoscopists (1, 2 and 3) performed all EMRs or ESDs and three pathologists (1, 2 and 3) diagnosed most of the cases. Transfer notes, medical records and endoscopic pictures of these cases were retrospectively reviewed and analyzed.

RESULTS: Adenocarcinoma was 72 (13.0%) cases in total 554 cases after endoscopic treatment of referred adenoma. When the grade of dysplasia was high (55.0%), biopsy number was more than three (22.7%), size was no smaller than 2.0 cm (23.2%), morphologic type was depressed (35.8%) or yamada type Ⅳ (100%), and color was red (30.9%) or mixed-or-undetermined (25.0%), it had much more malignancy rate than the others ($P < 0.05$). All 18 cases diagnosed as adenocarcinoma in the re-endoscopic forceps biopsy were performed by endoscopist 1. There were different malignancy rates according to the pathologist ($P = 0.027$).

CONCLUSION: High grade dysplasia is the most important factor for predicting malignancy as a final pathologic diagnosis before treating the referred gastric adenoma. This discrepancy can occur mainly through inappropriately selecting a biopsy site where cancer cells do not exist, but it also depends on the pathologist to some extent.

Key words: Discrepancy; Adenoma; High grade dysplasia; Endoscopic mucosal resection; Endoscopic submucosal dissection

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INTRODUCTION

Since gastric adenoma can progress to higher grade dysplasia or cancer, as shown in long-term follow up studies, it should be treated by endoscopic resection or surgical resection[1-3]. Endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD) have been approved as standard treatments for gastric adenoma[4]. Pathologic results from the mucosectomy specimens taken from endoscopic resection of gastric adenoma can be different from those of an endoscopic forceps biopsy[5,6]. As endoscopy has been examined more commonly and extensively, prevalence of adenoma referred from a primary care center for endoscopic resection has increased. However, there have been no reports on the histologic discrepancy between the endoscopy-based diagnosis of the referred gastric adenoma and the final pathologic diagnosis, and previous studies did not include analysis of other possible factors for discrepancy[5-7]. This study aimed to elucidate and analyze possible factors affecting discrepancy for referred gastric adenoma.

MATERIALS AND METHODS

A total of 1049 patients with gastric adenoma were endoscopically treated by EMR or ESD between July 2006 and June 2009 at Chungnam National University Hospital. Among these, 534 patients were referred from primary care centres, most of them from the Tae-jeon Chungchoeng province in South Korea. Because it was intended for all the referred patients to undergo endoscopic treatment, most patients were treated with EMR or ESD, except for an extreme few who had a tendency toward bleeding, or were untreatable due to size, location, or comorbidity. Endoscopists decided resection methods (EMR or ESD) from clinical information such as age, size, morphology, color, location and pathologic grade, but there were no strict criteria. Transfer notes, medical records, and endoscopic pictures of these cases were retrospectively reviewed and analyzed. One hundred and forty-two cases were examined by re-endoscopy and 108 cases underwent re-biopsy prior to endoscopic resection according to the judgment of the endoscopist. The main reason for pre-evaluations of endoscopic examination and biopsy before the resection was incomplete or confusing referred medical records for determining treatment methods. This is schematically described in Figure 1. Transfer reports were reviewed for information of histologic grade, biopsy number, date of the biopsy, and the name of the referring center. Pathologic reports on 54 patients were written as mild, moderate, or severe grade dysplasia of the adenoma, instead of low or high grade dysplasia. Grading terms of adenoma required unification for statistical analysis. "Mild grade"or "moderate grade" was classified as low grade and "severe grade" or "moderate to severe grade" was classified as high grade. Three endoscopists performed all EMRs or ESDs and three pathologists diagnosed most of the cases. Endoscopic reports and saved pictures of procedures were reviewed for morphologic type, color, size, and location.

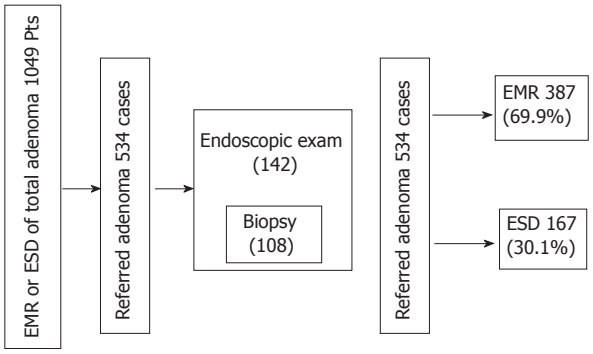

Figure 1 Schematic description of the study design. EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; Pts: Patients.

SPSS version 13.0 was used for statistic analysis. The one-way analysis of variance test was used for comparison of continuous variables; for example, age, size, day duration and biopsy number. The χ^2 test was used for other parameters of nominal variables.

RESULTS

Baseline characteristics, endoscopic features and treatment results of referred adenoma

Baseline characteristics and endoscopic features of referred adenomas from primary care centres are shown in Table 1. The mean age of the 554 cases was 66.1 years old. More than 86.4% of cases were located within and under the lower body. Results showed adenomas with no grading record in the transfer note in 92 cases (16.6%), low grade adenomas in 382 cases (69.0%), and high grade adenomas in 80 cases (14.4%). Treatment results of referred adenoma are shown in Table 2. Early gastric cancers were found in 72 cases (13.0%), no adenomatous lesions were found in 56 cases (10.1%), low grade adenomas were found in 356 cases (64.3%), and high grade adenomas were found in 68 cases (12.3%). One case involved mucosa associated lymphatic tissue lymphoma (MALToma) and one complicated case of bleeding were referred. Histologic results of pre-procedure re-endoscopic biopsy were various, from gastritis to adenocarcinoma. In the re-endoscopic biopsy, there were 18 cases (16.7%) of adenocarcinoma and one case of MALToma. The most common complication of EMR and ESD was bleeding (14 cases, 2.5%) which is defined as a case requiring an endoscopic procedure for bleeding control. Perforation (2 cases, 0.4%) and stricture (2 cases, 0.4%) were rare complications of EMR or ESD. There was one case of positive resection margin, in which surgery was

Table 1 The baseline characteristics and endoscopic features of referred adenomas *n* (%)

No. of cases	554
No. of patients	534
Age (yr), mean ± SD	62.1 ± 9.6
Male:female	372:182 (2.04:1)
Histologic grade	
Adenoma (no grading)	92 (16.6)
Low grade	382 (69.0)
High grade	80 (14.4)
No. of referring hospitals	116
No. of Bx, mean ± SD	2.24 ± 1.75
Mean duration between biopsy and procedure	40.7 d
Information of endoscopic photo	449 (81.0)
Size (cm), mean ± SD	1.2 ± 0.8
Morphologic type	
Elevated	275 (49.6)
Flat	206 (37.2)
Depressed	67 (12.1)
Y-Ⅳ	6 (1.1)
Color	
Whitish	332 (59.9)
Reddish	94 (17.0)
Mixed or undetermined	128 (23.1)
Longitudinal location	
Antrum	298 (53.8)
Angle	57 (10.3)
Body	191 (34.4)
High	12 (2.2)
Middle	55 (9.9)
Lower	124 (22.4)
Cardia or fundus	8 (1.5)
Circular location	
Anterior	123 (22.2)
Posterior	119 (21.5)
Lesser curvature	193 (34.8)
Greater curvature	113 (20.4)

Bx: Biopsy; Y-Ⅳ: Yamada type Ⅳ.

Table 2 Treatment results of referred adenomas *n* (%)

Repeat endoscopy	142 (25.6)
Repeat biopsy	108 (19.5)
No. of biopsy, mean ± SD	2.4 ± 1.0
Histologic results of repeat biopsy	
Low grade adenoma	73 (67.6)
High grade adenoma	9 (8.3)
Adenocarcinoma	18 (16.7)
Gastritis	7 (6.5)
Others	1 (0.9) (MALToma)
Endoscopist	
1	462 (83.4)
2	64 (11.6)
3	28 (5.1)
Pathologist	
1	340 (61.4)
2	124 (22.4)
3	83 (15.0)
Others	8 (1.1)
Histologic type; tubulovillous adenoma	10 (1.8)
Type of procedure	
EMR	387 (69.9)
ESD	167 (30.1)
Histologic results of post-procedure	
Low grade adenoma	356 (64.3)
High grade adenoma	68 (12.3)
EGC	72 (13.0)
No adenomatous lesion	56 (10.1)
Others	2 (0.4) (MALToma: 1, transfer by Cx: 1)
Complication	
Bleeding	14 (2.5)
Perforation	2 (0.4)
Stricture	2 (0.4)
Cases with multiple adenoma	
2 adenoma in a patient	12 patients/534 (2.2)
3 adenoma in a patient	4 patients/534 (0.7)

MALToma: Mucosa associated lymphoid tissue lymphoma; EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; EGC: Early gastric cancer; Cx: Complication.

performed for completion of treatment. Sixteen patients had multiple adenomas, 12 patients had 2 adenomas and 4 patients had 3 adenomas (Table 2).

Agreement and discrepancy of histologic diagnosis

Comparison of histologic diagnoses between local clinic endoscopic biopsy and repeat endoscopic biopsy and post-procedure specimens are described in Table 3. The rate of discrepancy between primary care center and repeat biopsy was 42.4% (39 cases/92), 38.1% (176 cases/462) between primary care center and post procedure specimens, and 29.6% (32 cases/108) between repeat biopsy and post procedure specimens. The rate of complete agreement was 57.6% (53 cases/108), 61.9% (286 cases/554), and 70.4% (76 cases/108), respectively. In all comparisons, the discrepancy rate of high grade dysplasia was higher than that of other forms of adenomas.

Although the histologic diagnosis of referred adenoma was as low grade dysplasia, it could be high grade (11.0%) or adenocarcinoma (5.8%) in the post procedure. High grade adenoma of the primary care center could also be low grade adenoma (27.5%) or early gastric cancer (55.0%) as a final pathologic diagnosis. Consistent high grade adenoma was only 15.0% between local clinics and post procedure biopsy. All adenocarcinomas of repeat endoscopic biopsies were early gastric cancer in the post procedure, except for one case, which had no adenomatous lesion. When this one case was reviewed with pathologists, the specimen from the repeat endoscopic biopsy was not enough for adenocarcinoma. There were only a few atypical glands, but this could still be suggestive of malignancy (Table 3).

Detection of adenocarcinoma in re-endoscopic repeat biopsy prior to the procedure

Histologic results of re-endoscopic biopsy (108 cases) are shown in (Table 4), according to the endoscopists and pathologists. All of the adenocarcinoma biopsies were performed by endoscopist 1 ($P < 0.001$). Pathologist 1 diagnosed a much larger number of adenocarcinomas than pathologist 2 ($P = 0.048$).

Risk factors for predicting malignancy of referred adenoma

There was no difference between the malignancy group

Table 3 Comparisons of histologic diagnoses *n* (%)

	LG	HG	EGC	NAL	Others	Total	Agreement	Discrepancy
Between local clinic biopsy and repeat biopsy								
Adenoma	11 (68.8)	3 (18.8)	0 (0)	1 (6.3)	1 (6.3)	16 (100)		
LG	51 (83.6)	4 (6.6)	2 (3.3)	4 (6.6)	0 (0)	61 (100)	51 (83.6)	10 (16.4)
HG	11 (35.5)	2 (6.5)	16 (51.6)	2 (6.5)	0 (0)	31 (100)	2 (6.5)	29 (93.5)
Total	73 (67.6)	9 (8.3)	18 (16.7)	7 (6.5)	1 (0.9)	108 (100)	53 (57.6)	39 (42.4)
Between local clinic and post procedure								
Adenoma	60 (65.2)	14 (15.2)	6 (6.5)	11 (12.0)	1 (1.1)	92 (100)		
LG	274 (71.7)	42 (11.0)	22 (5.8)	43 (11.3)	1 (0.3)	382 (100)	274 (71.7)	108 (28.3)
HG	22 (27.5)	12 (15.0)	44 (55.0)	2 (2.5)	0 (0)	80 (100)	12 (15.0)	68 (85.0)
Total	356 (64.3)	68 (12.3)	72 (13.0)	56 (10.1)	2 (0.4)	554 (100)	286 (61.9)	176 (38.1)
Between repeat forcep biopsy and post procedure								
LG	52 (71.2)	1 (15.1)	6 (8.2)	3 (4.1)	1 (1.4)	73 (100)	52 (71.2)	21 (28.8)
HG	3 (33.3)	4 (44.4)	2 (22.2)	0 (0)	0 (0)	9 (100)	4 (44.4)	5 (45.6)
Adenocarcinoma	0 (0)	0 (0)	17 (94.4)	1 (5.6)	0 (0)	18 (100)	17 (94.4)	1 (5.6)
Benign lesion	3 (42.9)	0 (0)	2 (28.6)	2 (28.6)	0 (0)	7 (100)	2 (28.6)	5 (71.4)
Others	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	1 (100)	1 (100)	0 (0)
Total	58 (53.7)	15 (13.9)	27 (25.0)	6 (5.6)	2 (1.9)	108 (100)	76 (70.4)	32 (29.6)

LG: Low grade; HG: High grade; NAL: No adenomatous lesion; EGC: Early gastric cancer.

Table 4 Results of re-endoscopic forceps biopsy according to endoscopists and pathologists *n* (%)

	LG	HG	Ade	NAL	Others	Total
Endoscopist						
1	34 (54.0)	6 (9.5)	18 (28.6)	4 (6.3)	1 (1.6)	63
2	36 (92.3)	3 (7.7)	0	0	0	39
3	3 (50.0)	0	0	3 (50.0)	0	6
Pathologist						
1	40 (64.5)	4 (6.5)	15 (24.2)	2 (3.2)	1 (1.6)	62
2	20 (66.7)	4 (13.3)	2 (6.7)	4 (13.3)	0	30
3	8 (80.0)	1 (10.0)	1 (10.0)	0	0	10
Others	5 (83.3)	0	0	1 (16.7)	0	6
Deletion of minority						
	Adenocarcinoma		Non-adenocarcinoma			*P* value
Endoscopist						
1	18 (28.6)		45 (71.4)			< 0.001
2	0 (0)		39 (100)			
Pathologist						
1	15 (24.2)		47 (77.8)			0.048
2	2 (6.7)		15 (93.3)			

Ade: Adenocarcinoma; LG: Low grade; HG: High grade; NAL: No adenomatous lesion.

and the non-malignancy group as a final pathologic diagnosis with regard to age, sex, histologic type, duration between local clinic biopsy and procedure, longitudinal and circular location, endoscopist, local clinics, and multiplicity. There was a difference with regard to histologic grade, number of biopsies, size, morphologic type, color, type of procedure, examination of repeat endoscopy, pathologist, and complications (Table 5).

Before the resection, predictive factors for a malignant result were high grade dysplasia (55.0%), a biopsy number of more than three (22.7%), a size of no less than 2.0 cm (23.2%), a morphologic type of depressed (35.8%) or yamada type Ⅳ (100%), and a red (30.9%) or mixed-or-undetermined (25.0%) coloration. There was no statistical significance between less than 1.0 cm and no less than 1.0 cm ($P = 0.124$).

Cases of ESD, repeat endoscopy, or complicated cases had many more malignant results than cases of EMR or direct procedures without re-endoscopy or non-complicated cases. The rate of malignancy was different according to the pathologist ($P = 0.027$). Mean duration from local clinic biopsy to endoscopic treatment did not differ between the malignancy group and the non-malignancy group. There was also no difference between cases (26 cases) with duration of no more than 14 d and cases (33 cases) of duration of more than 90 d. High grade dysplasia showed the highest odds ratio (19.5) with regard to risk factors for malignancy (Table 6).

DISCUSSION

Histologically, gastric adenomas are composed of cells with hyperchromatic, elongated nuclei arranged in a picket-fence pattern with cystic glands and nuclear atypia being occasionally present[8,9]. The malignant potential of adenomas has been demonstrated in long term follow up studies, even in low grade dysplasia, therefore, resection is recommended[3,10]. Since the introduction of EMR in Japan, techniques for endoscopic resection have been continuously advancing; therefore, EMR and ESD are now approved for use in standard treatment of gastric adenoma[4,11,12].

Predictive factors for malignancy

In univariate analysis, risk factors for malignant transformation included location, histologic type (tubulovillous), redness, and high grade dysplasia in the study by Park *et al*[5], and depressed type, high grade dysplasia, redness, ulceration in the study by Jung *et al*[6] in the univariate analysis. In multivariate analysis, only high grade dysplasia had a significant relationship with malignant transformation in the two studies. In our study, predictive factors for

Table 5 Comparison of the non-malignancy group and the malignancy group *n* (%)

	Non-malignancy group	Malignancy group	*P* value
No. of cases	482 (87.0)	72 (13.0)	
No. of patient	462 (86.5)	72 (13.5)	
Age (yr), mean ± SD	61.8 ± 9.8	63.7 ± 9.0	0.132
Sex			
Male	320 (86.0)	52 (14.0)	0.350
Female	162 (89.0)	20 (11.0)	
Histologic grade			
Adenoma	86 (93.5)	6 (6.5)	
Low grade	360 (94.2)	22 (5.8)	< 0.001
High grade	36 (45.0)	44 (55.0)	
Histologic type			
Tubulovillous	7 (70)	3 (30)	0.129
Tubular	475 (83.3)	69 (12.7)	
No. of Bx, mean ± SD	2.1 ± 1.6	2.9 ± 2.2	< 0.001
No. of biopsy			
Undetermined	111 (86.0)	18 (14.0)	
1	47 (92.2)	4 (7.8)	
2	117 (94.4)	7 (5.6)	
3	105 (92.1)	9 (7.9)	
4	47 (72.3)	18 (27.7)	
5	25 (80.6)	6 (19.4)	
6	12 (70.6)	5 (29.4)	
7	1 (33.3)	2 (66.7)	
8	1 (33.3)	2 (66.7)	
3	269 (93.1)	20 (6.9)	< 0.001
4	86 (72.3)	33 (27.7)	
Mean duration between Bx and procedure (d)	40.9	39.3	0.869
Duration between biopsy and procedure			
14 d	24 (92.3)	2 (7.7)	0.658
90 d	29 (87.9)	4 (12.1)	
Size (cm), mean ± SD	1.2 ± 0.8	1.5 ± 1.1	0.003
< 1.0	201 (89.7)	23 (10.3)	
> 1.0, < 2.0	218 (87.9)	30 (12.1)	0.010
> 2.0	63 (76.8)	19 (23.2)	
Morphologic type			
Elevated	252 (91.6)	23 (8.4)	
Flat	187 (90.8)	19 (9.2)	< 0.001
Depressed	43 (64.2)	24 (35.8)	
Y-Ⅳ	0 (0)	6 (100)	
Color			
Whitish	321 (96.7)	11 (3.3)	
Reddish	65 (69.1)	29 (30.9)	< 0.001
Mixed or undetermined	96 (75.0)	32 (25.0)	
Longitudinal location			
Antrum	255 (85.6)	43 (14.4)	
Angle	47 (82.5)	10 (17.5)	0.291
Body	173 (90.6)	18 (9.4)	
Cardia or fundus	7 (87.5)	1 (12.5)	
Circular location			
Anterior	104 (84.6)	19 (15.4)	
Posterior	107 (89.9)	12 (10.1)	0.573
Lesser curvature	171 (88.6)	22 (11.4)	
Greater curvature	95 (84.1)	18 (15.9)	
Type of procedure			
EMR	371 (95.9)	16 (4.1)	< 0.001
ESD	111 (66.5)	56 (33.5)	
Repeat endoscopy			
Yes	110 (77.5)	32 (22.5)	< 0.001
No	372 (90.3)	40 (9.7)	
Endoscopist			
1	397 (85.6)	65 (14.1)	
2	60 (93.8)	4 (6.3)	0.204
3	25 (89.3)	3 (10.7)	
Pathologist			
1	284 (83.5)	56 (16.5)	
2	117 (94.4)	7 (5.6)	0.027
3	74 (89.2)	9 (10.8)	
Others	6 (100)	0 (0)	
Local clinics (> 30 cases)			
1	39 (95.1)	2 (4.9)	
2	37 (90.2)	4 (9.8)	0.224
3	35 (97.2)	1 (2.8)	
4	28 (84.8)	5 (15.2)	
Complication			
Bleeding	9 (64.3)	5 (35.7)	
Perforation	1 (50)	1 (50)	
Stricture	1 (50)	1 (50)	
Total complications	11 (61.1)	7 (38.9)	0.005
No complications	471 (87.9)	65 (12.1)	
Multiplicity			
Patient of single case	449 (86.7)	69 (13.3)	0.464
Patient of multiple cases	13 (81.3)	3 (18.8)	

Bx: Biopsy; Y: Yamada type; EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection.

Table 6 Odds ratio of risk factors for malignancy as a final diagnosis

	Odds ratio
High grade dysplasia	19.5
Biopsy number ⩾ 4	5.1
Size ⩾ 2.0 cm	2.4
Depressed or Y-Ⅲ, Y-Ⅳ	7.3
Reddish or undetermined	11.1
ESD	11.7
Repeat endoscopy	2.7
Pathologist 1	2.4
Complications	4.6

ESD: Endoscopic submucosal dissection; Y: Yamada type.

malignancy as a final diagnosis included histologic grade, biopsy number, size, morphologic type and color. High grade dysplasia was the most important risk factor for malignancy, as in previous studies[5,6], with the highest odds ratio (Table 6).

Three out of ten cases (30%) of tubulovillous adenoma were malignancies, compared to only 69/475 cases (12.7%) of tubular adenoma, although this was not statistically significant. Cases with more than three biopsies were more often malignant than cases with fewer biopsies. This might be explained by the assumption that the endoscopist has taken more biopsies when he suspected a malignancy. ESD, re-endoscopy, and complicated groups had more many malignancies than EMR, direct procedure without re-endoscopy, and non-complicated groups, but those are not the cause of malignancy, but the result of strict treatment. Other possible factors affecting malignancy will be discussed below.

Possible causes affecting malignant discrepancy: (1) geographic variety of histology; forceps biopsy can be done only on the adenoma site, when cancer cells are mixed in the same lesion; (2) chronological difference between the time of forceps biopsy and the time of

resection; adenoma can be transformed to malignancy; (3) different criteria of pathologist with regard to malignancy; and (4) different location between forceps biopsy and resection.

Geographic variety of histology

Because relatively small forceps biopsy foci of the polyp cannot represent the entire lesion, there can be a discrepancy between the forceps biopsy and resection specimen of the polyp[13]. Discrepancies before and after endoscopic resection in adenoma are mainly due to the geographic distribution of malignant cells within the adenoma[5,6,14], which means that the discrepancy depends on the location of the initial endoscopic forceps biopsy. It is noteworthy that all of the adenocarcinomas (18 cases) in the re-endoscopic forceps biopsy before the procedure were performed by endoscopist 1, although there was no difference in the discrepancy rate between primary care center and post-treatment according to the endoscopist (Table 4). This may be due to the experience of the endoscopist. An expert endoscopist who can reduce the rate of discrepancy has the ability to determine the location of the cancer cells grossly, approximately to the real histology. A similar two studies on malignant transformation of adenoma presented different discrepancy rates in spite of similar study designs[5,6]. The rate of malignant transformation of adenoma was 6.8% (8/118) in the study by Park *et al*[5] and 55.3% (63/114) in the study by Jung *et al*[6]. These large differences can be understood in the same context.

Chronological difference between the time of forceps biopsy and the time of resection

Gastric adenoma can progress to early gastric cancer, as shown in long term follow-up studies[3,10,15]; even low grade dysplasia has malignant potential. This change can occur over a long period of time. Yamada *et al*[3] reported on one case of 37 low grade dysplasia and one case of 10 high grade dysplasia that progressed to invasive carcinoma over a period of 212 mo and 55 mo, respectively. In our study, duration from the time of initial biopsy to the time of resection was not different between the malignant group and the nonmalignant group. Statistically, the rate of malignancy was also not different between fewer than two weeks (7.7%, 2 cases/33) and fewer than 90 d (12.1%, 4 cases/26) in duration. This means that a treatment delay of roughly three months is not a problem.

Different criteria of pathologist with regard to malignancy

Because criteria between Japanese and Western pathologists are different, international workshops have been steadily and persistently organized in an effort to establish a consensus[16-18]. In 1996, eight pathologists from Japan and Western countries met in Tokyo and individually reviewed a set of 35 gastric biopsy and resection specimens of lesions with potential early neoplasias[16]. There was agreement between Japanese and Western viewpoints in only 11 of the 35 specimens. A different diagnosis can be made for the same specimen, even by an intraobserver in the time interval of three years[19]. Table 5 shows that the malignant discrepancy rate of pathologist 1 is approximately three times greater than that of pathologist 2. The rate of adenocarcinoma diagnosis for re-endoscopic forceps biopsy is also higher for pathologist 1 than pathologist 2 (Table 4). Although the forceps biopsy specimen was not reviewed, it can be assumed that forceps biopsy by the primary care center can be underestimated by the pathologist. However, no difference in the malignant discrepancy rate was observed between primary care centers (Table 5). It is a limitation of our study that the same specimens were not reviewed by pathologists.

Different location between forceps biopsy and resection

Logically, it is possible that either the patient or the sample changed, or that a different mucosectomy site was selected from the diagnostic biopsy site; however, this was not included in the discussion.

COMMENTS

Background

Endoscopic examination is performed more commonly in the primary care center, and gastric adenoma is more frequently referred to tertiary care units.

Research frontiers

There have been so many embarrassing events when previous and post-procedure diagnoses have been different. This research is performed to predict the treatment result and to discover the reasons for these events.

Innovations and breakthroughs

There have been no reports about the discrepancy of referred gastric adenoma and diverse predictive factors, and possible causes are included in this study.

Applications

The results of this study will help endoscopists to predict the results of treatment and to decide the proper treatment option.

Peer review

The authors studied various predictive factors for discrepancy of gastric adenoma and deeply analyzed possible causes of discrepancy.

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BRIEF ARTICLE

Vitamin D supplementation improves sustained virologic response in chronic hepatitis C (genotype 1)-naïve patients

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Abstract

AIM: To determine whether adding vitamin D, a potent immunomodulator, improves the hepatitis C virus (HCV) response to antiviral therapy.

METHODS: Seventy-two consecutive patients with chronic HCV genotype 1 were randomized into two groups: the treatment group (n = 36, 50% male, mean age 47 ± 11 years) received Peg-α-2b interferon (1.5 μg/kg per week) plus ribavirin (1000-1200 mg/d) together with vitamin D3 (2000 IU/d, target serum level > 32 ng/mL), and the control group (n = 36, 60% male, mean age 49 ± 7 years) received identical therapy without vitamin D. HCV-RNA was assessed by real-time polymerase chain reaction (sensitivity, 10 IU/mL). The sustained virologic response (SVR) was defined as undetectable HCV-RNA at 24 wk post-treatment.

RESULTS: Clinical characteristics were similar in both groups. The treatment group had a higher mean body mass index (27 ± 4 kg/m^2 *vs* 24 ± 3 kg/m^2, P < 0.01), viral load (50% *vs* 42%, P < 0.01), and fibrosis score (> F2: 42% *vs* 19%, P < 0.001) than the controls. At week 4, 16 (44%) treated patients and 6 (17%) controls were HCV-RNA negative (P < 0.001). At week 12, 34 (94%) treated patients and 17 (48%) controls were HCV-RNA negative (P < 0.001). At 24 wk post-treatment (SVR), 31 (86%) treated patients and 15 (42%) controls were HCV-RNA negative (P < 0.001). Viral load, advanced fibrosis and vitamin D supplementation were strongly and independently associated with SVR (multivariate analysis). Adverse events were mild and typical of Peg-α-2b/ribavirin.

CONCLUSION: Adding vitamin D to conventional Peg-α-2b/ribavirin therapy for treatment-naïve patients with chronic HCV genotype 1 infection significantly improves the viral response.

Key words: Hepatitis C; Vitamin D; Sustained viral response; Genotype 1; Fibrosis

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INTRODUCTION

The current treatment for hepatitis C virus (HCV) infection is pegylated interferon α combined with ribavirin

(Peg/RBV) administered for 24 wk for HCV genotypes 2 or 3, or 48 wk for HCV genotype 1, the most prevalent genotype in Israel, Europe, and North America[1]. The aim of HCV therapy is a sustained virologic response (SVR), defined as an undetectable serum HCV-RNA level at 24 wk after the cessation of therapy. For patients with HCV genotype 1, the rate of SVR ranges between 38% and 46%[2,3]. In subgroups of this population (e.g., Hispanics and African-Americans), the rate of SVR is even lower, reaching only 19%[4]. These differences are not explained by baseline viral load or compliance to treatment. Recent efforts to improve patient outcomes have focused on adding new antiviral therapies specifically targeted to HCV, including inhibitors of either HCV polymerase or protease[5]. However, few studies have addressed the issue of improving the host factors.

Vitamin D is a potent immunomodulator[6,7]. Increased production of 1, 25-dihydroxy vitamin D3 results in the synthesis of cathelicidin, a peptide capable of destroying many viral infectious agents as well as *M. tuberculosis*. Low serum levels of 25-hydroxyvitamin D (< 20 ng/mL) prevent macrophages from initiating this innate immune response, which may explain why African-Americans, who are often vitamin D deficient, are more prone to contracting tuberculosis and viral infections than Caucasians[8]. Moreover, vitamin D improves insulin sensitivity[9], suppresses proinflammatory cytokines, increases anti-inflammatory cytokines, and improves CD4 T cell hyperresponsiveness[10]. Vitamin D deficiency is very common (92%) among patients with chronic liver disease, and at least one-third suffer from severe vitamin D deficiency (< 12 ng/mL)[11]. Israeli subjects from various ethnic backgrounds are at higher risk of vitamin D deficiency[12]. Pettas and co-workers recently showed a low serum vitamin D level to be related to severe fibrosis and low responsiveness to interferon-based therapy in genotype 1 chronic hepatitis C (CHC)[13]. Its role and relationship to SVR and therapy in CHC are unknown. We reasoned that adding vitamin D to conventional therapy could improve treatment efficacy at weeks 4 [rapid viral response (RVR)] and 12 [early viral response (EVR)] during therapy, and 24 wk after cessation of therapy (SVR).

MATERIALS AND METHODS

Subjects

Study inclusion criteria were age 18-65 years, a chronic HCV genotype 1 infection, no previous treatment for hepatitis C, seronegative for HBV, HDV, and human immunodeficiency virus infections, an absolute neutrophil count of > 1500 per mm^3, a platelet count of > 90 000 per mm^3, and a normal hemoglobin level. Liver biopsies were required within 2 years prior to study entry, and the samples were examined by two pathologists who were unaware of patient identity and treatment regimen. The severity of hepatic inflammation and fibrosis was evaluated by the Ishak score in separate reports for grading and staging[14]. Exclusion criteria were decompensated liver disease (cirrhosis with a Child-Pugh score > 9), another cause of clinically significant liver disease, or the presence of hepatocellular carcinoma.

Study design

This was an intention-to-treat prospective randomized study. The experimental procedures were approved by the institutional review boards of the two participating medical centers. Informed consent was obtained from all participants (Clinical Trial Gov: NCT00804752)

The study included 72 consecutive CHC genotype 1 treatment-naïve patients who were stratified according to ethnic group (i.e., Russian/Jewish/Arab) due to possible differences in vitamin D levels. They were randomly assigned to one of two study groups. The treatment group comprising 36 patients (mean age 47 ± 11 years, 50% male) who received pegylated (peg)-interferon-α-2b (1.5 μg per kg body weight) plus oral ribavirin 1000 mg/d (for body weight < 75 kg) or 1200 mg/d (for body weight > 75 kg) and vitamin D3 (Vitamidyne D, Fischer Pharmaceuticals, Israel) 2000 IU/d, target serum level > 32 ng/mL) for 48 wk. Vitamin D3 was given by oral drops for 4 wk before the initiation of antiviral treatment and after serum levels had reached > 32 ng/mL in all patients in the treatment group. The supplemented vitamin D levels were maintained during the course of therapy with the same dosage as in the lead-in phase. The control group of 36 patients (mean age 49 ± 7 years, 60% male) received peg-interferon-α-2b (1.5 μg/kg body weight) plus ribavirin (1000-1200 mg/d) without vitamin D3 for 48 wk.

Efficacy assessments

Plasma HCV-RNA levels were measured using the COBAS Taq Man HCV assay, version 1.0 (Roche Molecular Systems), with a lower limit of quantification of 35-45 IU/mL and a lower limit of detection of 10 IU/mL. HCV-RNA levels were measured at the time of screening and during the treatment period at weeks 4, 12 and 48. All subjects had at least one follow-up visit at 24 wk after the completion of treatment. Those who had undetectable HCV-RNA levels had another follow-up visit 24 wk later, at which time HCV-RNA levels were measured again. Treatment efficacy was defined as SVR, i.e., undetectable HCV-RNA at 24 wk post-treatment. Clearance of HCV-RNA by real-time polymerase chain reaction (RT-PCR) was assessed at week 4 (RVR), week 12 (complete EVR), and at week 48 of treatment response (early treatment response, ETR). Patients with ETR who tested HCV-RNA positive during follow-up were classified as relapsers. Breakthrough was defined as an increase in the HCV-RNA level of one $\log_{10}$ unit compared with the lowest value. Therapy was discontinued if quantitative HCV-RNA levels at week 12 dropped by < 2 log compared with baseline values (non-responders), and at week 24 if HCV-RNA was still detectable in those patients in whom HCV-RNA dropped > 2 log at week 12[3,15].

Table 1 Baseline demographic, clinical and virologic characteristics of all patients

Baseline demographics	Peg/RBV (n = 36)	Peg/RBV + Vit D (n = 36)	P value
Age (yr)	49 ± 7	47 ± 11	0.123
Males (%)	60	50	0.015
Body mass index (kg/m^2)	24 ± 3	27 ± 4	0.014
HCV genotype: 1a/1b (n)	3/33	3/33	0.138
Baseline HCV-RNA (log IU/mL)	6.2 ± 0.8	6.1 ± 0.7	0.126
High viral load HCV-RNA > 800 000 IU/mL	15 (42%)	18 (50%)	0.033
Baseline ALT (U/L)	56 ± 31	55 ± 28	0.587
Advanced fibrosis (> F2)	7 (19%)	15 (42%)	0.001
Ethnicity (Russian /Jewish/Arab)	28/6/2	29/3/4	0.194

Peg/RBV: Pegylated interferon α and ribavirin; Vit D: Vitamin D; ALT: Alanine aminotransferase; HCV: Hepatitis C virus.

Safety assessments

Biochemical assessments were performed at each visit during the treatment period and at the post-treatment follow-up visit. Data on adverse events were collected and physical examinations were also performed each time. The safety assessment included complete blood count, antinuclear antibody, and thyroid-stimulating hormone levels. Peg-interferon α 2b was reduced to 1.0 μg/kg body weight in patients with a < 750 neutrophil count and withdrawn temporarily in patients with a < 500 neutrophil count. The same dose reduction was applied if platelet levels fell under 50 000 cells/mm^3, with peg-interferon being discontinued when the 25 000 cell/mm^3 threshold was reached. In both treatment arms, the ribavirin dose was tapered by 200 mg/d in patients with a hemoglobin level < 10 g/dL, and discontinued altogether in patients with a level < 8.0 g/dL.

Clinical and laboratory measurements

Vitamin D levels: 25 (OH)-vitamin D3 levels were determined by 125 I-radioimmunoassay (Dia-Sorin, Stillwater, MN, United States)[16]. 25-OH vitamin D is the major circulating form of vitamin D and is used as an indicator of vitamin D status. Vitamin D deficiency was defined as a 25 (OH)-vitamin D serum level < 12 ng/mL, vitamin D insufficiency as 25 (OH)-vitamin D levels of 12-32 ng/mL, and vitamin D sufficiency as levels > 32 ng/mL[12]. Insulin resistance was estimated using the homeostasis model assessment (HOMA-IR)[17]. HOMA-IR was measured at baseline and at 4 wk in both study groups. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. Obesity was defined as a BMI exceeding 28 kg/m^2. C-reactive protein was determined by the nephelometric method[18]. Paraoxonase activity was measured according to a method using phenylacetate as a substrate[19]. α tocopherol (vitamin E) was estimated spectrophotometrically[20]. Malondialdehyde concentration was estimated spectrophotometrically using the thiobarbituric acid assay[21]. Calcium, phosphor, vitamin B12, thyroid-stimulating hormone, glucose, insulin, liver enzymes, albumin, bilirubin, prothrombin time, and creatinine were measured by standard biochemical tests.

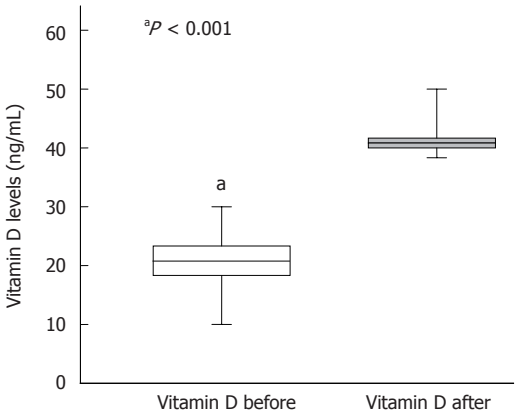

Figure 1 Vitamin D serum levels before and at 4 wk after the beginning of antiviral treatment + vitamin D supplementation (n = 36). Bars represent standard error.

Statistical analysis

Results were expressed as mean ± SD. The difference between the two groups was assessed by the chi-squared test for categorical variables and by the Mann-Whitney rank test for continuous variables. The Spearman correlation was used to express correlations between variables. The primary study endpoint was evidence of the influence of vitamin D on the viral response at weeks 4 and 12 during therapy and at week 24 post-treatment. Logistic regression analysis was performed to detect independent predictors for SVR. The significance level was set at $P < 0.05$. The statistical analyses were carried out with the WINSTAT (Kalmia, CA, United States) software program.

RESULTS

At baseline, 21% of the patients in the treatment group had severe vitamin D deficiency (< 12 ng/mL), 59% had insufficiency, and 20% had sufficient vitamin D levels. The control group baseline tests showed that 27% had vitamin D deficiency, 60% had insufficiency, and 13% had sufficient vitamin D levels. Table 1 shows the clinical and biochemical parameters of the patient populations.

The treatment group had higher BMI levels, viral loads, and fibrosis scores > F2 than the controls (27 ± 4 kg/m^2 *vs* 24 ± 3 kg/m^2, $P = 0.014$; > 800 000 IU/mL, 50% *vs* 42%, $P = 0.033$; 42% *vs* 19%, $P = 0.001$, respectively). There were no differences between the two groups in terms of age, HCV genotype, baseline HCV-RNA, ethnic background, or aminotransferases levels.

Figure 1 depicts the baseline and week 4 vitamin D levels at the beginning of antiviral therapy. Serum vitamin D levels were significantly lower at baseline (20.5 ± 9.0 ng/mL) and increased after 4 wk of vitamin D treatment to a mean level of 37 ± 10 ng/mL. Baseline

Table 2 Viral response, vitamin D levels, biomarkers of inflammation, insulin resistance, and oxidative stress in all patients

Parameter	Peg/RBV (n = 36)	Peg/RBV + Vit D (n = 36)	P value
Viral response			
Relapser	13 (36%)	3 (8%)	0.001
Non-responder	8 (22%)	2 (6%)	0.010
HOMA-IR			
Baseline	4.6 ± 5.7	4.5 ± 1.4	0.123
After 4 wk	5.0 ± 4.0	2.3 ± 1.0[a]	0.001
Basal vitamin	19 ± 6	20.5 ± 9.0	0.177
D-25-OH levels (ng/mL)			
Malondialdehyde (mmol/L)	0.11 ± 0.05	0.13 ± 0.04	0.810
Paraoxonase (mmol/L/min)	0.57 ± 0.1	0.64 ± 0.1	0.120
Vitamin E (μg/mL)	19.7 ± 8.8	21 ± 8.0	0.510
Vitamin B12 pmol/L	316 ± 190	331 ± 170	0.103
CRP mg/dL	0.39 ± 0.3	0.45 ± 0.4	0.100
Triglycerides (mg/dL)	200 ± 80	220 ± 60	0.110

[a]P < 0.001 between HOMA-IR at baseline and HOMA-IR after 4 wk of treatment with vitamin D. HOMA-IR: Homeostasis model assessment of insulin resistance; Peg/RBV: Pegylated interferon α and ribavirin; Vit D: Vitamin D; CRP: C-reactive protein.

vitamin D levels were also lower in the control group (19 ± 6 ng/mL, Table 2).

Figures 2 and 3 show the rates of RVR, EVR, and SVR in the treatment and control groups. At week 4, 16 (44%) patients in the treatment group and 6 (17%) controls were HCV-RNA negative, and at week 12, 34 (94%) and 17 (48%), respectively, were HCV-RNA negative (P < 0.001 for each week). Twenty-four weeks after the cessation of therapy (SVR), 31 (86%) patients in the treatment group and 15 (42%) controls were HCV-RNA negative (P < 0.001). The percentage of relapses and non-responders and the biomarkers of insulin resistance, inflammation, pro-oxidant levels, antioxidant levels, and baseline vitamin D, vitamin E, and vitamin B12 serum levels are shown in Table 2 for both groups.

The rate of viral breakthrough was null. The rates of relapse and non-response were significantly lower in the treatment group compared with the control group [n = 3 (8%) *vs* n = 13 (36%), P < 0.001, and 2 (6%) *vs* 8 (22%), P < 0.01, respectively]. The HOMA-IR index decreased significantly after 4 wk of treatment with vitamin D compared with the control group (from 4.5 ± 1.4 to 2.3 ± 1.0, P < 0.01 *vs* 4.6 ± 5.7 to 5.0 ± 4.0, respectively, P < 0.1). There was no difference between groups for malondialdehyde, paraoxonase, vitamin E, vitamin B12, C-reactive protein, and triglyceride levels.

The adherence to vitamin D treatment was excellent during the entire course, and all patients in the treatment group achieved the target level. Vitamin D levels were maintained during the course of therapy with the same dosage (2000 IU/d) as in the lead-in phase. Predictive factors for SVR in patients treated with Peg/RBV combination therapy are shown in Table 3.

Viral load, advanced fibrosis, baseline vitamin D levels, changes in HOMA-IR, and vitamin D supplementation were significant univariate predictors of SVR. Viral load, vitamin D supplementation, advanced fibrosis and changes in HOMA-IR remained as independent predictors in the multivariate analysis. Thus, vitamin D supplementation emerged as being more responsible for higher SVR than the baseline vitamin D level.

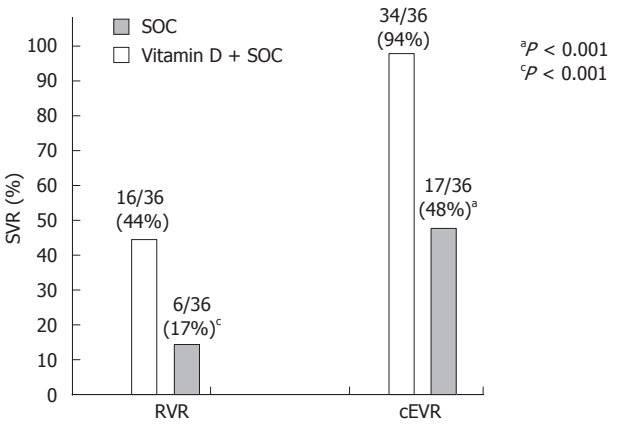

Figure 2 Rate (%) of the rapid viral response and rate of early viral response in the treatment (n = 36) and control (n = 36) groups. RVR was defined as undetectable HCV RNA at 4 wk during treatment. Complete EVR (cEVR) was defined as undetectable HCV RNA at 12 wk during treatment. SOC: Standard of care; RVR: Rapid viral response; EVR: Early viral response; HCV: Hepatitis C virus; SVR: Sustained viral response.

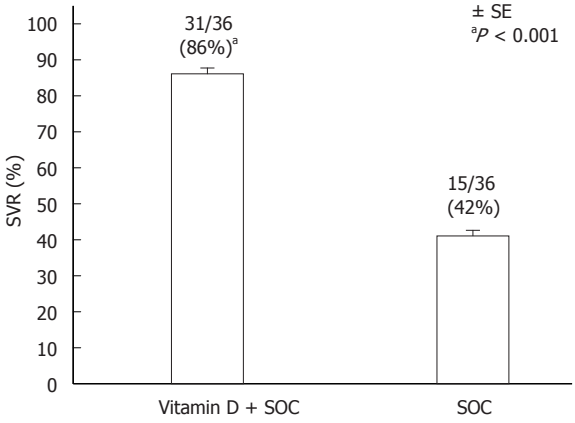

Figure 3 Rate of sustained viral response in the treatment group (Vitamin D + SOC, n = 31/36) and the control group (n = 15/36, SOC) 6 mo after cessation of treatment. SVR was defined as undetectable HCV-RNA at 24 wk post-treatment. Bars represent standard error. SOC: Standard of care; SVR: Sustained viral response; HCV: Hepatitis C virus.

The most common adverse events were mild in nature, similar in both groups, and consistent with typical Peg/RBV-induced systemic symptoms. They included nausea (n = 4), headache (n = 4), insomnia (n = 5), chills (n = 4), myalgia (n = 3), pyrexia (n = 3), pruritus (n = 2), mild neutropenia (n = 3), mild thrombocytopenia (n = 5), and mild anemia (n = 3). There were no serious adverse events. Adherence to Peg/RBV combination therapy was excellent, and there was no difference in dose reduction Peg/RBV combination therapy due to adverse events in either group. No patient discontinued treatment. Changes in laboratory values during the study were consistent with those reported in association with

Table 3 Predictors for sustained virologic response in treatment-naïve hepatitis C virus genotype 1 patients with pegylated interferon α and ribavirin combination therapy

	Odds ratio	95% CI	*P* value
Vitamin D treatment (Yes *vs* No)	2.5	2.0-4.9	< 0.001
Baseline vitamin D (< 20 or > 20 IU/mL)	1.5	1.2-3.8	0.080
Advanced fibrosis (< F2 or > F2)	2.0	1.0-3.6	0.001
High viral load (< 800 000 or > 800 000 IU/mL)	2.8	1.2-4.0	0.001
Baseline CRP (< 0.05 or > 0.5 mg/dL)	1.0	0.5-1.9	0.510
Changes in homeostasis model assessment (%)	1.8	0.5-3.0	0.030

CRP: C-reactive protein; CI: Confidence interval.

the combined use of Peg/RBV[3].

DISCUSSION

The results of this study suggest that the addition of a vitamin D supplement to current standard therapy can significantly improve the rate of RVR, EVR and SVR in treatment-naïve patients with HCV genotype 1 compared the rates with standard therapy alone. The observed SVR in the control group (42%) was consistent with previous reports[2,3]. Overall there was a marked increase in the virologic response at week 4 (44% *vs* 17%), week 12 (94% *vs* 48%), and week 24 after the cessation of therapy (86% *vs* 42%), and a low rate of relapse (8% *vs* 36%) with vitamin D supplementation compared with no supplementation. The rate of relapse in the control group was within the reported 18%-40% range for current standard HCV antiviral therapy[2,22].

There are only two reports examining the association between vitamin D status and outcome of antiviral therapy in patients with chronic HCV viral infection. Petta and co-workers retrospectively analyzed a cohort of 167 patients treated with Peg/RBV for hepatitis C, and detected an association between lower vitamin D serum levels and failure to achieve SVR[23]. Our results provide further support for that data. The second study by Bitetto and co-workers showed that vitamin D supplementation improved the response to antiviral treatment for recurrent HCV in liver transplant recipients[24]. Several differences between those two studies should be noted. Bitetto and co-workers' HCV patients were immunocompromised, and they were supplemented with low-dose vitamin D (800 IU/d) after liver transplantation. In addition, most of their HCV patients (75%) had low vitamin D levels despite treatment. Finally, that study was retrospective and focused on the prevention of osteoporosis, not on the treatment of hepatitis C.

The exact mechanism of action leading to improved RVR, EVR, and SVR in patients receiving vitamin D is unknown. Vitamin D is metabolized by the liver and converted to 1,25-dihydroxy-vitamin D3, which is the active form of the vitamin[6,7]. Individuals with chronic liver disease may have poor conversion from vitamin D3 or any of its other biologically active metabolites[11]. 1,25 vitamin D3 appears to modulate immunity principally *via* regulation of T-cell function[25]. The vitamin D receptor (VDR) is expressed on virtually every type of cell involved in immunity[26]. The immunomodulatory actions of vitamin D are elicited through its direct action on T-cell antigen-presenting cell function[27]. T helper cell type 1 (TH1) actions are intensified when vitamin D is insufficient, as in the majority of our patient population, or when signals through VDR are weak. Regulatory T cell and TH2 cells are diminished, thus favoring an autoimmune TH1 response[28]. This is a pro-inflammatory response which may impair IFN and insulin signaling, thus decreasing the viral response[29,30]. A recent study on 120 patients with chronic HCV genotype 1 infections reported that a TH1 to TH2 ratio of < 15.5 was significantly associated with SVR (odds ratio 9.6)[31]. TH1 and TH2 measurements were not performed in the present study. Persistent HCV infection modulates the balance between immune stimulatory and inhibitory cytokines which can prolong inflammation and lead to fibrosis and chronic liver diseases[32]. More recently, Gutierrez and co-workers showed that vitamin D3 increased VDR protein expression and inhibited viral replication in cell culture[33].

It is well known that people of African and Hispanic descent are less likely to respond to standard therapy[34]. This may be due to a polymorphism of the *interleukin (IL)-28B* gene, polymorphism of VDR or vitamin D deficiency[13,35]. The vast majority of the Russian/Jewish/Arab patients in the present study had vitamin D insufficiency, possibly related to paradoxically low exposure to the sun in this predominantly sunny country and/or to a low supply of vitamin D from their diet.

The impact of diet on liver fibrosis and on response to IFN therapy in patients with HCV chronic hepatitis has been reported before[36]. HCV patients also lack vitamins E and B12[37,38]. A recent study showed that higher levels of vitamin B12 were associated with SVR, but there was no difference in serum levels of those vitamins between the group treated with vitamin D and the controls[39].

Insulin resistance emerged as one of the most important host factors in the prediction of the response in non-diabetic HCV patients treated with Peg/RBV, and is a common factor in the features associated with difficult-to-treat patients[40]. Vitamin D is also known to help prevent type 2 diabetes, and it is possible that low levels of vitamin D lead to insulin resistance[9]. The direct effect of vitamin D may be mediated by binding of its circulating active form to the pancreatic B cell vitamin D receptor[41]. Vitamin D deficiency or insufficiency may alter the balance between the extracellular and intracellular cell calcium pools, which may interfere with normal insulin release[42]. Thus, a lack of either calcium or vitamin D can result in peripheral insulin resistance[41]. Moreover, oxidative stress leeches calcium, and vitamin

D helps absorb calcium[43]. Our current results confirm these findings: the HOMA-IR was higher at baseline in the vitamin D treatment group and improved after 4 wk of therapy compared to the control group. Moreover, the changes in HOMA-IR were strongly associated with SVR (multivariate analysis).

The definition of normal vitamin D serum levels is a subject of debate. In the current study, increasing the vitamin level D to > 32 ng/mL increased the response to antiviral therapy to the same extent in patients with vitamin D deficiency as well as those with vitamin D insufficiency. Multivariate analysis revealed that viral load, advanced fibrosis and vitamin D supplementation remained as independent predictors. Thus, it can be concluded that vitamin D supplementation is responsible for a higher SVR, rather than the baseline vitamin D level. It remains to be determined whether the addition of vitamin D acts by a mechanism other than improvement of insulin resistance or immune function such as the upregulation of toll-like receptors involved in the immune response in HCV-infected patients

Limitations of the present study include the small number of patients, lack of vitamin D level assessment during therapy for the treatment and control groups, and that this prospective and randomized study was not placebo-controlled, thus the patients knew whether or not they received a vitamin D supplement. Another limitation is the lack of data on the TH1 and TH2 immune response. The identification of determinants of the response, such as polymorphisms of the *IL-28B* gene, polymorphism of the VDR and immune function[13,35], may help explain the difference in response rates between patients with different ethnic backgrounds. This was not done in our study since data on IL-28B and on VDR polymorphism were not available at the time the study was designed.

In conclusion, the addition of vitamin D to Peg/RBV combination therapy in treatment-naïve patients who were infected with HCV genotype 1 significantly increased the rates of rapid, early, and sustained viral responses.

COMMENTS

Background

Treating chronic hepatitis C virus (HCV) (genotype1) patients with pegylated interferon and ribavirin, which is considered to be the standard of care, has achieved viral clearance in less than 50% of the patients. Vitamin D is a potent immunomodulator with a beneficial effect against viral and bacterial infections. The vast majority of patients with chronic hepatitis C have low levels of vitamin D. Different new drugs such as protease or polymerase inhibitors are still under investigation and are expensive and have many side effects like rash.

Research frontiers

Vitamin D deficiency is well documented in patients with chronic liver disease. However, treating patients with chronic HCV infection by adding a vitamin D supplement to the standard of care has not been addressed. There are only two reports dealing with the association between vitamin D status and outcome of antiviral therapy for chronic HCV infection.

Innovations and breakthroughs

The current study shows that adding a vitamin D supplement to pegylated interferon and ribavirin significantly increases the rapid, early and late clearance of the virus, in chronic hepatitis C genotype 1 treatment-naïve patients.

Applications

This study emphasizes the importance of vitamin D supplementation when added to standard treatment in all patients with chronic hepatitis C. Further studies are needed to explain the mechanism of vitamin D supplementation for these patients.

Terminology

Hepatitis C is a chronic liver infection that can be complicated by liver failure and liver cancer. Clearance of the virus from the blood is achievable by a combination of pegylated interferon and ribavirin in less than 50% of the patients. Vitamin D has an important role in the treatment of different bacterial and viral infections; this vitamin is synthesized in the skin by absorption of ultraviolet from the sun light. The mechanism of action of this vitamin is unknown, but it may improve the activities of immune cells that are important in the eradication of HCV.

Peer review

This is a well conducted study with a relevant finding, and it is well written.

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BRIEF ARTICLE

Posterior lingual lidocaine: A novel method to improve tolerance in upper gastrointestinal endoscopy

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Abstract

AIM: To evaluate the effect of posterior lingual lidocaine swab on patient tolerance to esophagogastroduodenoscopy, the ease of performance of the procedure, and to determine if such use will reduce the need for intravenous sedation.

METHODS: Eighty patients undergoing diagnostic esophagogastroduodenoscopy in a tertiary care medical center were randomized to either lidocaine swab or spray. Intravenous meperidine and midazolam were given as needed during the procedure.

RESULTS: Patients in the lidocaine swab group (SWG) tolerated the procedure better than those in the spray group (SPG) with a median tolerability score of 2 (1, 4) compared to 4 (2, 5) ($P < 0.01$). The endoscopists encountered less difficulty performing the procedures in the SWG with lower median difficulty scores of 1 (1, 5) compared to 4 (1, 5) in the SPG ($P < 0.01$). In addition, the need for intravenous sedation was also lower in the SWG compared to the SPG with fewer patients requiring intravenous sedation (13/40 patients *vs* 38/40 patients, respectively, $P < 0.01$). The patients in the SWG were more satisfied with the mode of local anesthesia they received as compared to the SPG. In addition, the endoscopists were happier with the use of lidocaine swab.

CONCLUSION: The use of a posterior lingual lidocaine swab in esophagogastroduodenoscopy improves patient comfort and tolerance and endoscopist satisfaction and decreases the need for intravenous sedation.

Key words: Esophagogastroduodenoscopy; Upper gastrointestinal endoscopy; Local anesthesia; Lidocaine; Sedation

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INTRODUCTION

Esophagogastroduodenoscopy (EGD) is an essential and very commonly used procedure for the evaluation of a

multitude of gastrointestinal (GI) symptoms including abdominal pain, hemorrhage, dysphagia, odynophagia, and reflux[1-3]. Although EGD is fairly safe, it carries a low risk of complications including perforation, bleeding, infection, and medication reactions/adverse effects[2,4,5]. Several studies showed that patients with advanced age and those with cardiopulmonary disease may carry an increased risk for the procedure especially when high doses of intravenous (IV) sedatives are used[2,6,7]. Various IV agents such as midazolam, meperidine, propofol, and fentanyl have been used over the past few decades for their anxiolytic, amnestic, and analgesic effects during the procedure[8-10]. However, these agents carry potential serious adverse effects especially in high risk patients. These complications include apnea, hypoxia, vomiting, hypotension, agitation, and allergic reactions[11-16]. In addition, complications of IV sedation contributed to cost increase due to unexpected hospitalizations and work related absenteeism on the procedure day. This has led to search for modes of anesthesia that carry less complication rates and, at the same time, provide satisfaction for both patient and endoscopist[12,15,17-24]. Few studies have used different forms of topical anesthesia including, spray, lollipop, and inhaler with mixed results. Some of these topical agents still carried a risk of retching, vomiting, and apnea[8,19,20,23,25-28]. Conventionally, topical lidocaine spray is used combined with IV analgesics and sedatives before and during the procedure to achieve a high level of patient comfort and endoscopist satisfaction[4,19,26,27,29].

The rationale behind the use of topical anesthesia is to suppress the gag reflex that may account for some of the EGD-related discomfort. The gag reflex is one of the normal reflexes induced by stimulation of the pharynx and velar area. It involves the contraction of pharyngeal constrictors induced by touching one of the five trigger zones that include: base of tongue, uvula, palate, posterior pharyngeal wall, and palatopharyngeal and palatoglossal folds[30]. The gag reflex consists of an afferent and an efferent arches. The afferent receives input from nerve fibers of the glossopharyngeal nerve which are relayed in the nucleus solitaris. The efferent arch is supplied by the nucleus ambiguus through the vagus nerve (Figure 1)[31]. These nuclei are at close proximity to the vomiting and salivating centers, which explains the experience of retching and excessive salivation when the gag reflex is elicited[30]. Both superficial and deep sensory receptors are involved in the physiology of the gag reflex, and this makes a pharyngeal plexus block superior to topical lidocaine spray in suppressing the reflex[32]. When a person eats, central voluntary action on the pharyngeal muscles dominates over the gag reflex and this is why there is no gagging when eating[30]. Therefore, if lidocaine is to be applied specifically to the above-mentioned five trigger areas in the pharynx, then the gag reflex would be markedly attenuated or even ablated during the procedure, which may further increase the patients' tolerance to EGD and in turn decrease IV sedation use. The use of lidocaine in the gel form may be ideal since a dense/sticky form of lidocaine may provide a more reliable local anesthesia compared to the spray.

In this study, the efficacy of posterior lingual lidocaine as a potential anesthetic technique in patients undergoing EGD was compared to that of the conventional lidocaine spray. Our main objective is to evaluate the effect of posterior lingual lidocaine application on patient tolerance to the procedure and the ease of performance of the procedure. Our secondary aim is to determine if such use will reduce the need for IV sedation.

MATERIALS AND METHODS

Patients

Our target population was patients undergoing diagnostic EGD for various indications at the American University of Beirut-Medical Center (AUB-MC). The study was approved by the Institutional Review Board committee at AUB-MC in accordance with Helsinki Declaration. The details regarding the study objectives and risks were fully explained to the patients and those who agreed to participate in the study were recruited and signed the informed consent.

Study design

After signing the informed consent, patients were randomly assigned to one of two study groups: the swab group (SWG) who received 150 mg of lidocaine gel or the spray group (SPG) who received 300 mg lidocaine spray. Lidocaine spray was administered using the same technique in 3 consecutive 30-s intervals, each consisting of 10 sprays (10 mg/dose) of Xylocaine® Pump Spray 10% (AstraZeneca AB, Sodertalje, Sweden). In the swab group, Xylocaine® Jelly 2% (AstraZeneca AB, Sodertalje, Sweden), with a lidocaine concentration of 20 mg/mL was used. A total of 7.5 mL (150 mg) of lidocaine gel was gradually applied to the base of the tongue and the peritonsillar areas. The endoscopist was totally blinded to the randomization. The endoscope used in the procedures was GIF-1T 240 (Olympus Optical, 11 mm diameter, Tokyo, Japan). All the patients had IV lines inserted and their vital signs (blood pressure, heart rate, respiratory rate) and pulse oxymetry were continuously monitored during the procedure. The data that was collected by the research fellow from all enrolled patients before the procedure included the following parameters: age, gender, past medical history, past surgical history, medications, allergies, alcohol use, smoking, illicit drug use, and history of previous endoscopy (including tolerance to it). None of the participants had any severe pulmonary disease (asthma and chronic obstructive pulmonary disease). The research fellow then determined the patients' anxiety level according to a scale from 1 to 5 (1 = no anxiety to 5 = extreme anxiety). After the topical anesthetics were applied, the time for the onset of the topical anesthesia was also noted (from the time the local anesthetic was applied till patients reported numb-

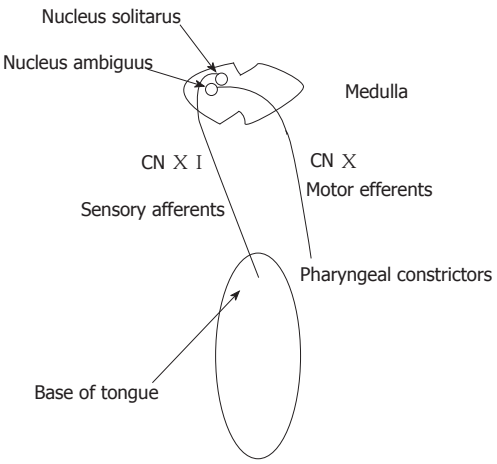

Figure 1 The gag reflex pathway: afferent fibers from the trigger areas in the pharynx and tongue carried by the glossopharyngeal nerve (cranial nerve X I) to the nucleus solitarus which sends the input to the nucleus ambiguus in the medulla oblongata. Efferent fibers from the nucleus ambiguus carried *via* the vagus nerve (CN X) to the pharyngeal constrictors to contract and cause gagging. CN: Cranial nerve.

ness in the oral cavity and the inability to swallow).

In both study groups, the decision to administer IV sedation during the procedure was made by the endoscopist depending on the patient's tolerance and the presence or absence of signs of discomfort, like excessive gag, retching, or restlessness. Sedatives used were midazolam and meperidine. The duration of the procedure was also noted.

Endoscopist's assessments

After the administration of the local anesthetics, the endoscopist rated the gag reflex based on a scale from 1 to 5 (1 = absent to 5 = strong). After the procedure, the endoscopist determined the ease of the procedure based on a scale from 1 to 5 (1 = easy to 5 = difficult). Finally, the amount of IV sedation given was recorded.

Patients' assessments

After the procedure was concluded, patients were monitored in the recovery room. Afterwards, a questionnaire was filled in by the participants to determine tolerance to the procedure based on a scale from 1 to 5 (1 = no difficulties encountered to 5 = very difficult). Also, symptoms during (retching, nausea, vomiting, abdominal pain, dyspnea, cough) and after (sore throat, nausea, vomiting, abdominal pain, dyspnea, cough) the procedure were recorded. Patients were also asked to specify the most uncomfortable phase of the procedure and their willingness to repeat the procedure using the same local anesthetic.

Statistical analysis

Statistical analysis was performed using SPSS for Windows version 16.0 (SPSS, Inc., Chicago, IL). The nonparametric Mann-Whitney test was used to compare ordinal variables (such as the gag reflex, procedure evaluation, *etc*.) and data that are not normally distributed (the doses of meperidine and midazolam) between SWG and SPG groups.

The χ^2 test was utilized to compare categorical variables between the 2 groups. Continuous variables were assessed with an independent sample *t* test. A P value < 0.05 was considered to be significant.

Table 1 Patient characteristics

	SWG (n = 40)	SPG (n = 40)	P value
Gender (M/F)	12/28	19/21	0.11
Mean age, yr (SD)	55.8 (18.1)	48.5 (18.2)	0.07
Smoking (yes/no)	15/25	23/17	0.07
Caffeine (yes/no)	37/3	32/8	0.19
Alcohol (yes/no)	12/28	14/26	0.63
Previous EGD (yes/no)	17/23	23/17	0.18

SWG: Swab group; SPG: Spray group; M: Male; F: Female; EGD: Esophagogastroduodenoscopy. P value for difference between groups using χ^2 or Fisher's exact tests.

Table 2 Pre-procedure evaluation, median (min, max)

	SWG (n = 40)	SPG (n = 40)	P value[1]
Anxiety	3 (1, 5)	3 (1, 5)	0.67
Time to onset of anesthesia (s)	80 (30, 300)	50 (20, 120)	< 0.01
Gag reflex	2 (1, 5)	4 (2, 5)	< 0.01

SWG: Swab group; SPG: Spray group. Anxiety rate: 1 = no anxiety to 5 = extreme anxiety; Gag reflex scale: 1 = absent to 5 = strong. [1]P value for difference between groups using nonparametric Mann-Whitney test.

RESULTS

Patient characteristics and demographics

Our study included 80 consecutive patients who underwent an elective EGD at AUB-MC. There were 31 males (38.8%) and 49 females (61.2%) with a mean age of 52.2 ± 18.4 years. There was no statistically significant difference in the patients' characteristics in both groups (Table 1).

Pre-procedure evaluation

Anxiety before the procedure was rated on an ascending scale from 1 to 5 as detailed in the method section. There was no significant difference between the two groups; the median anxiety scores were 3 (1, 5) for subjects in SWG and SPG.

The time interval between the lidocaine administration and the onset of anesthesia was significantly longer in the SWG as compared to the SPG, with median time 80 (30, 300) and 50 (20, 120) s (P < 0.01, respectively).

The SPG had significantly stronger gag reflex than the SWG with respective median scores of 4 (1, 5) and 2 (1, 5) (P < 0.01, Table 2).

IV sedation use

IV sedation was administered more frequently in the SPG than the SWG (95% *vs* 32%, P < 0.01).

Table 3 Use of intravenous sedation, median (min, max)

	SWG (n = 40)	SPG (n = 40)	P value
Use of IV sedation (yes/no)	13/27	38/2	< 0.01
Meperidine dose (mg)	0 (0, 50)	25 (0, 75)	< 0.01[1]
Midazolam dose (mg)	0 (0, 3)	2 (0, 4)	< 0.01[1]

SWG: Swab group; SPG: Spray group; IV: Intravenous. [1]P value for difference between groups using Mann-Whitney test.

Table 4 Procedure evaluation, median (min, max)

	SWG (n = 40)	SPG (n = 40)	P value
Ease of procedure - endoscopist	1 (1, 5)	4 (1, 5)	< 0.01
Procedure tolerance - patient	2 (1, 4)	4 (2, 5)	< 0.01[1]
Patient willingness to repeat procedure (yes/no)	32/8	2/38	< 0.01[1]

SWG: Swab group; SPG: Spray group. Endoscopist difficulty scale: 1= easy to 5 =difficult; Patient tolerance scale: 1 = no difficulties encountered to 5 = very difficult. [1]P value for difference between groups using nonparametric Mann-Whitney test.

The amount of meperidine administered was significantly lower in the SWG compared to the SPG, with median doses of 0 (0, 50) and 25 (0, 75) mg, respectively, $P < 0.01$. Similarly, the dose of midazolam was significantly lower in the SWG as compared to the SPG with median doses of 0 (0, 3) and 2 (0, 4) mg ($P < 0.01$, respectively, Table 3).

Endoscopists' evaluation

The endoscopist's assessment of the degree of procedure difficulty showed that the procedures were significantly easier to perform in the SWG than the SPG, with median difficulty scores of 1 (1, 5) and 4 (1, 5) ($P < 0.01$, respectively, Table 4).

Additionally, the procedure was significantly much easier to perform in subjects who did not receive IV sedation compared to those who received either meperidine or midazolam, with median difficulty scores of 1 (1, 3) and 4 (1, 5) ($P < 0.01$), respectively.

Patients' evaluation

Patients in the SWG tolerated the procedure more with a median tolerability score of 2 (1, 4) as compared to 4 (2, 5) in the SPG ($P < 0.01$, Table 4).

The most difficult part of the procedure was the introduction of the endoscope as reported by 68.8 % of patients. Thirty two (80%) subjects in the SWG expressed their willingness to repeat the procedure under the same local anesthesia, versus only 2 (5%) patients in the SPG ($P < 0.01$, Table 4).

Side effects

The side effects during and after the procedure were similar in both groups except for retching which was significantly lower in the SWG than in the SPG (13/40 *vs* 31/40 patients, respectively, $P < 0.01$, Table 5).

Table 5 Procedure-related symptoms

	SWG (n = 40)	SPG (n = 40)	P value
During the procedure			
Retching (yes/no)	13/27	31/9	< 0.01
Cough (yes/no)	10/30	12/28	0.62
Abdominal pain (yes/no)	1/39	1/39	1
Dyspnea (yes/no)	0/40	4/36	0.12
After the procedure			
Sore throat (yes/no)	5/35	8/32	0.55
Abdominal pain (yes/no)	1/39	5/35	0.2
Nausea/vomiting (yes/no)	0/40	1/39	1

SWG: Swab group; SPG: Spray group. P value for difference between groups using χ^2 or Fisher's exact tests.

Complications

None of the procedures was aborted due to complications, excessive agitation or major patient discomfort.

DISCUSSION

The use of conscious sedation along with lidocaine spray is the standard of care in upper GI endoscopy[4,19,26,27,29]. However, IV sedation may cause potential harm to the patients especially the elderly with co-morbidities. These side effects include hypotension, respiratory depression, and paradoxical agitation[7,11-16]. The potential risks of upper GI endoscopy are mostly related to the use of IV sedation[1,2,11,16,27,33-35]. Studies done by Campo *et al*[6] and Mulcahy *et al*[7] showed that a high level of anxiety, young age, and a strong gag reflex are risk factors for poor tolerance to upper GI endoscopy. On the other hand, a study done by Pereira *et al*[27] showed that patients' anxiety did not contribute to procedure tolerance. Local oropharyngeal anesthesia including lidocaine has been studied in several trials with the results showing that the use of the lidocaine spray or gel with IV sedation increased the tolerability and ease of the procedure and reduced the risk of discomfort during the procedure[23,26,28,29,36]. A single study, however, was done on the lidocaine lollipop which showed excellent efficacy in achieving patient comfort even without the use of IV sedation[28]. The action of local oropharyngeal anesthesia is achieved mainly by inhibiting the gag reflex which is one of the most important factors affecting the tolerability and ease of the procedure[6,28]. So in order to perform the procedure without possibly using IV sedation, an effective local agent that suppresses the gag reflex should be used.

Our study showed that when lidocaine gel is applied to the posterior lingual area, it effectively suppresses the gag reflex, significantly increases the patient tolerability to the procedure, improves endoscopist satisfaction of the procedure, and considerably decreases the need for IV sedation. The level of anxiety and age were similar in both groups; thus, these factors can be eliminated as confounding variables. Therefore, lidocaine gel could be

used as a sole agent in upper GI endoscopy sparing the use of IV sedation with its potential complications. In addition, this may help patients resume their daily activities immediately after the procedure. Although we did not compare the cost of the lidocaine gel and spray, the use of the gel appears to be more cost-effective since potential adverse events related to IV sedation are reduced. The maximal dose of lidocaine used in the spray group was 300 mg. This dose of lidocaine is within the recommended dose of 5 mg/kg and does not exceed the potentially toxic dose of 500 mg[37]. Moreover, higher doses of topical lidocaine had been used in prior studies. Sutherland *et al*[38], for instance, utilized topical doses of 380 mg of lidocaine and concluded that the blood levels were still within therapeutic range. The dose of lidocaine used in the gel group was much lower (150 mg) than that in the spray group. Despite that decrease in the dose, there was more effective suppression of the gag reflex in the gel group, and hence, better tolerance to the EGD.

Sample size was one of the few limitations in this study. Because it was a small sample, subgroup analysis could not be performed. Another limitation that might have affected our results can be attributed to the impairment in judgmental abilities caused by the sedatives used in some cases.

In conclusion, this study presented evidence that the use of lidocaine swab applied to the posterior lingual area was an effective mode of local anesthesia in upper GI endoscopy. This can lead to reduction in the use of IV sedatives (and potentially their complications) and may decrease the overall cost of the procedure. This may be a very promising modality especially in the elderly patients who have comorbidities, and in office-based upper GI endoscopy. However, larger, multicenter studies should be done to confirm and validate the results of our study.

ACKNOWLEDGMENTS

The data of this study has been presented as a poster at the Digestive Disease Week in New Orleans, 2010.

COMMENTS

Background

Esophagogastroduodenoscopy (EGD) has become an essential and very commonly used procedure for the diagnostic and therapeutic evaluation of a multitude of upper gastrointestinal (GI) symptoms and diseases. EGD is considered a safe procedure with a very low risk of complications. Medication administered for local anesthesia and for conscious sedation during the procedure can pose some adverse effects especially in the elderly population. So finding ways to decrease the need for these drugs would decrease the complication rates. The rationale behind the use of topical anesthesia is to decrease the gag reflex that may account for a major part of EGD-related discomfort. Using lidocaine as a topical anesthetic in the gel form may be ideal since a dense/sticky form of lidocaine may provide a more reliable local anesthesia compared to the spray thus increasing the patients' tolerance to EGD and in turn decreasing the need for intravenous (IV) sedation.

Research frontiers

Improving the tolerance and ease of execution of EGD procedures has gained much interest recently. The primary objective of this clinical research approach is to decrease the need for drugs used for conscious sedation to spare patients the side effects and the costs of elevated doses of such agents. Research is currently focusing on increasing the effectiveness of drug administration, improving patients' tolerance, and using/developing ultrathin endoscopes.

Innovations and breakthroughs

This study showed that when lidocaine gel is applied to the posterior lingual area, it effectively suppresses the gag reflex, significantly increases the patient tolerability to the procedure, improves endoscopist satisfaction of the procedure, and considerably decreases the need for IV sedation.

Applications

The authors presented evidence that the use of lidocaine swab applied to the posterior lingual area was an effective mode of local anesthesia in upper GI endoscopy. This can lead to reduction in the use of IV sedatives (and potentially their complications) and may decrease the overall cost of the procedure. This may be a very promising modality especially in the elderly patients with comorbidities, and in office-based upper GI endoscopy.

Terminology

Conscious sedation: Defined as moderate sedation by the American Society of Anesthesiologists. It is the reduction of irritability or agitation by administration of sedative drugs such as midazolam with purposeful preservation of the response to verbal or tactile stimulation. Posterior lingual lidocaine swab: A technique whereby local anesthesia is achieved by the application of lidocaine gel to the base of the tongue and the peritonsillar areas as opposed to application *via* the aerosolized spray form routinely utilized.

Peer review

This article showed that the effectiveness of the posterior lingual lidocaine swab is statistically significant. The study design and analysis ensures the validity of achieved results and nearly eliminated causes of random error. Nonetheless, increasing the patients' number and possibly involving other centers in this study would undeniably increase its power and reliability.

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BRIEF ARTICLE

Statin use and the risk of colorectal cancer: A population-based case-control study

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Abstract

AIM: To investigate whether the use of statins is associated with colorectal cancer risk.

METHODS: We conducted a population-based case-control study in Taiwan. Data were retrospectively collected from the Taiwan National Health Insurance Research Database. Cases consisted of all patients who were aged 50 years and older and had a first-time diagnosis of colorectal cancer between the period 2005 and 2008. The controls were matched to cases by age, sex, and index date. Adjusted odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using multiple logistic regression.

RESULTS: We examined 1156 colorectal cancer cases and 4624 controls. The unadjusted ORs for any statin prescription was 1.10 (95% CI = 0.94-1.30) and the adjusted OR was 1.09 (95% CI = 0.91-1.30). When statin use was categorized by cumulative dose, the adjusted ORs were 0.99 (95% CI = 0.78-1.27) for the group with cumulative statin use below 105 defined daily doses (DDDs); 1.07 (95% CI = 0.78-1.49) for the group with cumulative statin use between 106 and 298.66 DDDs; and 1.30 (95% CI = 0.96-1.75) for the group with cumulative statin use of 298.66 DDDs or more compared with nonusers.

CONCLUSION: This study does not provide support for a protective effect of statins against colorectal cancer.

Key words: Case-control study; Colorectal cancer; Pharmacoepidemiology; Statins

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INTRODUCTION

Statins are inhibitors of 3-hydroxy-3-methyl glutaryl coenzyme A reductase which is a key enzyme in the rate-limiting step in cholesterol synthesis[1]. Statins are commonly used as cholesterol-lowering medications and have shown effectiveness in the primary and secondary prevention of heart attack and stroke[2,3]. The extensive evidence in this field has led to widespread use of these drugs.

Rodent studies indicate that statins are carcinogenic[4]. In contrast, several recent studies on human cancer cell lines and animal tumor models indicate that statins may have chemopreventive properties through the arrest of cell cycle progression[5], induction of apoptosis[1,6], suppression of angiogenesis[7,8], and inhibition of tumor growth and metastasis[9]. Results of a meta-analysis and observational studies revealed either no association[10-17] or a decrease in cancer incidence[18-26]. The reasons for the varying results are unclear but may be related to methodological issues, including small sample size and short follow-up periods[27].

Several epidemiologic studies have investigated the association between statin use and risk of colorectal cancer and the results have been inconsistent. Ten studies reported no statistically significant association between statin use and colorectal cancer risk[10,12,15,17,20-21,27-30]. However, three recent case-control studies reported that statin use is associated with a significant reduction in the risk of colorectal cancer[22,31-32].

Since large numbers of people utilize statins on a long-term basis, and because epidemiologic data linking statin use and risk of colorectal cancer are conflicting, we undertook the present study in Taiwan to evaluate the association between statin use and colorectal cancer risk.

MATERIALS AND METHODS

Data source

The National Health Insurance (NHI) program, which provides compulsory universal health insurance, was implemented in Taiwan on March 1, 1995. Under the NHI, 98% of the island's population can receive all forms of health care services including outpatient services, inpatient care, Chinese medicine, dental care, childbirth, physical therapy, preventive health care, home care, and rehabilitation for chronic mental illness. In cooperation with the Bureau of NHI, the National Health Research Institute (NHRI) of Taiwan randomly sampled a representative database of 1 000 000 subjects from the entire NHI enrollees by means of a systematic sampling method for research purposes. There were no statistically significant differences in age, gender, and healthcare costs between the sample group and all enrollees, as reported by the NHRI. This dataset (from January 1996 to December 2008) includes all claim data for these 1 000 000 subjects, and offers a good opportunity to explore the relation between the use of statins and risk of colorectal cancer. These databases have previously been used for epidemiological research, and information on prescription use, diagnoses, and hospitalizations has been shown to be of high quality[33-35].

Because the identification numbers of all individuals in the NHRI databases were encrypted to protect the privacy of the individuals, this study was exempt from full review by the Institution Review Board.

Identification of cases and controls

Cases consisted of all patients who were aged 50 years and older and had a first-time diagnosis of colorectal cancer (International Classification of Diseases, 9th revision, Clinical Modification Code 153-154) over a 4-year period, from January 1, 2005 to December 31, 2008, and who had no previous diagnosis of cancer.

Controls comprised patients who were admitted to hospital for diagnoses that were unrelated to statin use including orthopedic conditions, trauma (excluding wrist and hip fractures), and other conditions (acute infection, hernia, kidney stones, cholecystitis)[12,36]. Wrist and hip fractures were excluded because previous studies have reported a reduced risk of osteoporosis among statin users[37-40]. We identified four control patients per case patient. Control patients were matched to the cases by sex, year of birth, and index date and were without a previous cancer diagnosis. For controls, the index date (date of hospital admission) was within the same month of the index date (date of first-time diagnosis of colorectal cancer) of their matched case.

Exposure to statins

Information on all statin prescriptions was extracted from the NHRI prescription database. We collected the date of prescription, the daily dose, the number of days supplied. The defined daily doses (DDDs) recommended by the WHO[41] were used to quantify use of statins. Cumulative DDDs were estimated as the sum of dispensed DDD of any statins (lovastatin, pravastatin, rosuvastatin, fluvastatin, simvastatin, or atorvastatin) from January 1, 1996 to the index date.

Potential confounders

For all individuals in the study population, we identified variables which might confound the associations between statin use and colorectal cancer, including diabetes mellitus, cholecystectomy, liver disease, colorectal polyps, and inflammatory bowel disease, recorded between January 1, 1996, and the index date. In addition, we also obtained

Table 1 Demographic characteristics of colorectal cancer cases and controls

Variable	Cases (n = 1156)	Controls (n = 4624)	Odds ratio (95% CI)
Age, yr (mean ± SD)	68.34 ± 10.40	69.29 ± 10.40	-
Female (%)	447 (38.67)	1788 (38.67)	-
No. of hospitalizations	0.29 ± 0.93	0.26 ± 0.74	P = 0.23
Diabetes (%)	422 (36.51)	1560 (33.74)	1.13 (0.99-1.29)
Cholecystectomy (%)	21 (1.82)	105 (2.27)	0.80 (0.50-1.28)
Liver disease (%)	422 (36.51)	1861 (40.25)	0.85 (0.75-0.98)
Colorectal polyps (%)	56 (4.84)	76 (1.64)	3.05 (2.14-4.33)
Inflammatory bowel disease (%)	82 (7.09)	315 (6.81)	1.04 (0.81-1.34)
Colonoscopy (%)	153 (13.24)	42 (0.91)	16.64 (11.75-23.57)
FOBT (%)	152 (13.15)	216 (4.67)	3.09 (2.48-3.84)
NSAID (%)	636 (55.02)	2767 (59.84)	0.82 (0.72-0.93)
Use of other lipid-lowering drugs (%)	31 (2.68)	180 (3.89)	0.68 (0.46-1.00)

FOBT: Fecal occult blood testing; NSAID: Non-steroidal anti-inflammatory drug; CI: Confidence interval.

prescription data for other lipid-lowering drugs (including fibrate, niacin, bile-acid binding resins, and miscellaneous medications) and non-steroidal anti-inflammatory drugs (NSAIDs) that could potentially confound the association between statin use and the risk of colorectal cancer. We defined users of the above-mentioned medications as patients with at least one prescription over one year prior to the index date. Furthermore, colonoscopy, fecal occult blood testing (FOBT), and number of hospitalizations one year before the index date were treated as confounders.

Statistical analysis

For comparisons of proportions, chi-square statistics were used. A conditional logistic regression model was used to estimate the relative magnitude in relation to the use of statins. Exposure was defined as patients who received at least one prescription for a statin at any time between January 1, 1996 and the index date. In the analysis, the subjects were categorized into one of four statin exposure categories: nonusers (subjects with no prescription for any statins at any time between January 1, 1996 and the index date), low (the lowest 50th percentile; ≤ 105 DDDs); medium (50th-75th percentile; 106-298.66 DDDs); and high (above the 75th percentile; > 298.66 DDDs) based on the distribution of use among controls. Odd ratios (ORs) and their 95% confidence intervals (CIs) were calculated using patients with no exposure as the reference. Analyses were performed using the SAS statistical package (version 8.02, SAS Institute Inc). All statistical tests were two-sided. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Records from 1156 colorectal cancer cases and 4624 selected matched controls were included in the analyses.

Table 2 Associations between statin use and colorectal cancer risk in a population-based case-control study, Taiwan, 2005-2008

	Cases (n)/ controls (n)	Crude OR (95% CI)	Adjusted OR (95% CI)[1]
Overall			
No statin use	914/3727	1.00	1.00
Any statin use	242/897	1.10 (0.94-1.30)	1.09 (0.91-1.30)
Cumulative use			
0	914/3727	1.00	1.00
1-105 DDD	112/451	1.02 (0.82-1.27)	0.99 (0.78-1.27)
106-298.66 DDD	60/221	1.11 (0.83-1.49)	1.07 (0.78-1.49)
> 298.66 DDD	70/225	1.27 (0.96-1.68)	1.30 (0.96-1.75)

OR: Odds ratio; CI: Confidence interval; DDD: Defined daily dose. [1]Adjusted for matching variable, diabetes, number of hospitalizations, cholecystectomy, liver disease, colorectal polyps, inflammatory bowel disease, colonoscopy, fecal occult blood testing, non-steroidal anti-inflammatory drugs and use of other lipid-lowering drugs.

Table 1 shows the distribution of demographic characteristics and selected medical conditions of the cancer cases and controls. The mean age was 68.34 years for cancer cases and 68.81 years for the controls. Case subjects were more likely to have had preventive services (screening colonoscopy and FOBT). The case group had a significantly higher rate of colorectal polyps than control patients. Use of other lipid-lowering drugs and NSAIDs were not significantly different between cases and controls.

The observed associations between the use of statins and colorectal cancer are shown in Table 2. Ever-use of any statins was associated with a slight but not statistically significant increased colorectal cancer risk (adjusted OR = 1.09, 95% CI = 0.91-1.30). When statin use was categorized by cumulative dose, the adjusted ORs were 0.99 (95% CI = 0.78-1.27) for the group with cumulative statin use below 105 DDDs; 1.07 (95% CI = 0.78-1.49) for the group with cumulative statin use between 106 and 298.66 DDDs; and 1.30 (95% CI = 0.96-1.75) for the group with cumulative statin use of 298.66 DDDs or more compared with nonusers. Overall, we found no association between cumulative statin use and colorectal cancer risk. ORs for cancers of the colon and rectum considered separately were similar (data not shown).

DISCUSSION

In this population-based case-control study, we found that statin drug use was not associated with colorectal cancer risk. Our findings are consistent with ten recent studies which reported no associations between statin use and overall colorectal cancer risk[10,12,15,17,20-21,27-30].

Our results, however, conflict with three recent case-control studies. In a case-control study conducted in Israel, a reduced risk of colorectal cancer was found to be associated with the use of statins for at least 5 years, compared with less than 5 years of use (OR = 0.50, 95% CI = 0.40-0.63)[22]. Another population-based study from Germany showed that statin use was associated

with a 35% (OR = 0.65, 95% CI = 0.43-0.99) colorectal risk reduction occurring within 1-4 years of statin use and no further risk reduction was seen after 5 years or more[31]. Neither study characterized the dose or duration of statins in detail and both studies defined statin use by recall. In a nested case-control study consisting solely of veterans with diabetes, using national databases of the Department of US Veterans Affairs and Medicare-linked files, Hachem *et al*[32] reported an odds ratio 0.91 (95% CI = 0.86-0.96) for colorectal cancer in relation to any statin use. However, there is no clear dose-response or duration-response relationship between filled statin prescriptions and colorectal cancer risk.

Duration of statin use may be important when investigating the chemopreventive effects of statins. We assessed exposure to statins measured as cumulative DDDs. Cumulative DDDs is a time-independent variable in which the daily supplies of each statin prescription dispensed were summed over time from January 1, 1996 to the index date. Because cumulative DDDs and statin duration are highly interrelated, it was not possible to model them together. Similar findings were noted when statin users were stratified by duration (data not shown).

There are at least two differences between our study and the study of Hachem *et al*[32]. First, their study population was limited to mostly male veterans with active access to health care and thus they were more likely to be prescribed a statin than the general population. Statin use was present in 51% of the study population. In our study this number was 19.4%. Second, the above-mentioned study was conducted among patients with diabetes who are known to have a higher likelihood of developing colorectal cancer[42]. Therefore, it is possible that it was easier to show benefit owing to the generally elevated risk in patients with diabetes[32]. Using an epidemiologic study which is restricted to patients with major risk factors means that the results of the restricted study may not necessarily apply to the portion of the population that was excluded. Whether a protective effect only occurs among patients who are already at higher risk of colorectal cancer requires further study. Other studies have reported a possible protective effect of statins in patients with diabetes on lung (adjusted OR = 0.43, 95% CI = 0.38-0.49)[24], pancreatic (adjusted OR = 0.32, 95% CI = 0.23-0.44)[25], and liver cancer (adjusted OR = 0.74, 95% CI = 0.64-0.87)[26].

One of the strengths of our study is the use of a computerized database, which is population-based and is highly representative. Because we included all patients newly diagnosed with colorectal cancer from 2005 to 2008, and because the control subjects in this study were selected from a simple random sampling of the insured general population, we can rule out the possibility of selection bias. Statins were available only on prescription. Because the data on statin use were obtained from an historical database which collects all prescription information before the date of colorectal cancer, recall bias for statin use was thus avoided.

Several limitations of the present study should be noted. First, although we adjusted for several potential confounders in the statistical analysis, a number of possible confounding variables, including family history of colorectal cancer, dietary habits or physical activity, and alcohol and tobacco use, which are associated with colorectal cancer were not included in our database. Second, we were unable to contact the patients directly about their use of statins because of anonymization of their identification number. Using pharmacy records representing dispensing data rather than usage data might have introduced an overestimation of statin use. However, there is no reason to assume that this would be different for cases and controls. Even if the patients did not take all of the statins prescribed, our findings would underestimate the effect of statin use. Third, lovastain and pravastatin (available in 1990), simvastatin (available in 1992), and fluvastatin (available in April, 1996) became available prior to patient enrollment in the database. Prescriptions for these drugs prior to 1996 would not be captured in our analysis. This could have underestimated the cumulative DDDs and may weaken the observed association. In addition, some exposure misclassification was likely caused by the fact that information on prescription was available only from 1996. Such misclassification, however, was likely to be non-differential, which would tend to underestimate rather than overestimate the association. Fourth, we were unable to analyze the risks for users of distinct statins separately due to the relatively small number of cases and the relatively small number of statin users. Fifth, data on the accuracy of discharge diagnoses are not available in Taiwan. Potential inaccurate data in the claims records could lead to possible misclassification. However, there is no reason to assume that this would be different for cases and controls. Lastly, as with any observational study, residual confounding by unmeasured factors which are different between cases and controls is also possible. However, the confounding effect of medical attention could be corrected for by introducing the number of hospitalizations into the conditional logistic regression model.

In summary, the results of this study do not provide support for an association between statin use and colorectal cancer risk. Given the widespread use of statins, it is prudent public health policy to continue monitoring cancer incidence among statin users, particularly as the duration of use is increasing[12].

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COMMENTS

Background

Experimental studies have shown that statins have potential protective effects against cancer. Several epidemiologic studies have investigated the association between statin use and risk of colorectal cancer, and the results are inconsistent.

Research frontiers

This study was undertaken to examine the relationship between statin use and the risk of colorectal cancer.

Applications

Statins are widely used cholesterol-lowering drugs, and the duration of use is increasing. Further and larger studies are needed to determine the long-term effects of statin use on cancer development and to clarify whether statins are truly effective for cancer chemoprevention.

Peer review

This is a nice population-based study which strength is the fact that Taiwan National Health Insurance research program provides extensive data of one million patients. The methodology and statistical analysis is adequate and altogether the authors provide convincing evidence that statin use does not protect against the risk of colorectal cancer.

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BRIEF ARTICLE

IκB kinase-beta inhibitor attenuates hepatic fibrosis in mice

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Abstract

AIM: To investigate the anti-fibrosis effect of IκB kinase-beta inhibitor (IKK2 inhibitor IMD0354) in liver fibrosis.

METHODS: Twenty male C57BL6 mice were divided into four groups. Five high-fat fed mice were injected with lipopolysaccharide (LPS, 10 mg/kg) intraperitoneally and five high-fat fed mice were without LPS injection to build models of liver injury, and the intervention group (five mice) was injected intraperitoneally with IKK2 inhibitor (IMD 30 mg/kg for 14 d), while the remaining five mice received a normal diet as controls. Hepatic function, pathological evaluation and liver interleukin-6 (IL-6) expression were examined. Western blotting and real-time polymerase chain reaction were used to detect the expressions of nuclear factor-κB (NF-κB), alpha-smooth muscle actin (α-SMA), tumor growth factor-beta1 (TGF-β1), tumor necrosis factor-alpha (TNF-α), type Ⅰ and type Ⅲ collagen proteins and mRNA.

RESULTS: A mouse model of liver injury was successfully established, and IMD decreased nuclear translocation of NF-κB p65 in liver cells. In the IMD-treated group, the levels of alanine aminotransferase (103 ± 9.77 μ/L *vs* 62.4 ± 7.90 μ/L, $P < 0.05$) and aminotransferase (295.8 ± 38.56 μ/L *vs* 212 ± 25.10 μ/L, $P < 0.05$) were significantly decreased when compared with the model groups. The histological changes were significantly ameliorated. After treatment, the expressions of IL-6 (681 ± 45.96 *vs* 77 ± 7.79, $P < 0.05$), TGF-β1 (Western blotting 5.65% ± 0.017% *vs* 2.73% ± 0.005%, $P < 0.05$), TNF-α (11.58% ± 0.0063% *vs* 8.86% ± 0.0050%, $P < 0.05$), type Ⅰ collagen (4.49% ± 0.014% *vs* 1.90% ± 0.0006%, $P < 0.05$) and type Ⅲ collagen (3.46% ± 0.008% *vs* 2.29% ± 0.0035%, $P < 0.05$) as well as α-SMA (6.19 ± 0.0036 μ/L *vs* 2.16 ± 0.0023 μ/L, $P < 0.05$) protein and mRNA were downregulated in the IMD group compared to the fibrosis control groups ($P < 0.05$).

CONCLUSION: IKK2 inhibitor IMD markedly improved non-alcoholic fatty liver disease in mice by lowering NF-κB activation, which could become a remedial target for liver fibrosis.

Key words: Liver fibrosis; IKK2 inhibitor; Nuclear factor-kappa B; Tumor growth factor-beta1; Interleukin-6; Alpha-smooth muscle actin; C57BL mouse

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INTRODUCTION

The incidence rate of non-alcoholic fatty liver disease (NAFLD) has increased annually. Simple steatosis in the

early stage may gradually develop into fatty hepatitis[1,2], and subsequently develop towards hepatic fibrosis and liver cirrhosis[3]. In an advanced stage, the incidence rate of liver cancer, multiple organ failure and other fatal complications reaches up to 0.6%-3%[4]. Based on the World Health Organization prognostication, chronic liver disease is the ninth leading cause of death in western countries and this situation will not be improved in the coming decades[5]. Among patients with non-alcoholic steatohepatitis (NASH), there were 10%-25% of patients that developed hepatic fibrosis or even liver cirrhosis[6-9]. The nosogenesis of NASH remains unclear, but the hypothesis of "secondary strike" has been widely accepted[10,11]. Unfortunately, some treatments initially gradually improve liver adipose degeneration, but are unable to achieve long-term control[9,12].

IκB kinase (IKK) is a large protein complex that is 700-900 kDa, including the two kinase subunits IKKα (IKK1) and IKKβ (IKK2) and one regulatory subunit that is either nuclear factor-kappa B (NF-κB) essential modifier or IKKγ. IKK2 is part of the inhibitor of the κB (IκB) IKK complex, which activates NF-κB through phosphorylation of the IκBs, leading to a series of inflammatory reactions[13-16]. Many effective drugs can reduce the inflammatory reaction in the liver by inhibiting the nuclear factor IKK2-NF-κB pathway and reducing insulin resistance in the liver[17]. Mathers *et al*[5] have demonstrated that application of the IKK2 inhibitor reduces fat accumulation in the liver and body weight gain in the mice. It has been reported that antioxidants inhibit the activity of NF-κB and can reduce inflammatory reactions[18] or even change fibrotic tissue[19].

We speculated that specific inhibition of NF-κB activation by an IKK2 inhibitor could effectively suppress the expression of inflammatory factors and even improve hepatic fibrosis. Therefore, in our study, a NASH model was established by a high-fat diet in mice and intraperitoneal injection of lipopolysaccharides (LPS) promoted acute hepatic injury and the expression of inflammatory factors. An IKK2 inhibitor (IMD0354) was used to suppress the NF-κB signaling pathway. Liver function and histological changes were observed and expression levels of interleukin-6 (IL-6), tumor growth factor-beta1 (TGF-β1), tumor necrosis factor-alpha (TNF-α), as well as other pro-inflammatory and pro-fibrosis factors, were determined. We focused on measurement of the fibrosis index, which represented hepatic stellate cells (HSCs), alpha-smooth muscle actin (α-SMA) and the expression levels of collagen I , collagen Ⅲ and mRNA, which showed fibrotic hepatic changes. Accordingly, we investigated potential therapeutic prospects of the IKK2-NF-κB signaling pathway for reversing fibrosis in NAFLD.

MATERIALS AND METHODS

Experimental protocol and animal model

Twenty-four-week-old male C57BL6 mice, weighing approximately 12-16 g, were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Science. Mice were housed in a clean grade barrier systems laboratory in the Medical Laboratory Animal Center of Shanghai Jiao Tong University. Animals were randomized into four groups: the control group (n = 5), high-fat (HF) diet group (n = 5), HF + LPS group (n = 5), and HF + LPS + IMD (IKK2 inhibitor) group (n = 5). The mice in the control group were given a normal diet (ND) and the HF group animals were fed with an HF diet for 10 wk. The ND chow was supplied by the Animal Center of the Medical College of Subsidiary Basic Medical of Shanghai Jiao Tong University. The HF diet (50% fat, pork fat 18%, yolk 12%, sugar 8% and basal diet 62%) was supplied by SLAC Precision Equipment Inc. Mice in the intervention group were intraperitoneally injected with 30 mg/kg IKK2 IMD 0354 (Tocris Bioscience, Bristol, United Kingdom) for 14 d, and at the end of 12 wk this was combined with intraperitoneal injection of 10 mg/kg LPS (Sigma-Aldrich, St Louis, MO, United States). Mice were then sacrificed after fasting for 12 h. Subsequently, 1 mL eyeball blood was obtained and all mice were killed by cervical dislocation. The liver tissue was fast fixed and lightly washed in ice-cold phosphate buffered solution (PBS). Then, part of the liver was placed into 10% formalin fixation solution, while the other part of the liver was quickly stored at -70 ℃ for cryopreservation. The blood was sent to the laboratory of Renji Hospital for liver enzyme assays. Some liver tissue was embedded in paraffin for 24 h and was then observed by hematoxylin and eosin (HE) staining, Masson staining and immunohistochemistry (IHC). Other liver tissue was saved under an ultra low temperature for Western blotting and polymerase chain reaction (PCR) procedures.

Biochemical and liver enzymes assays

Serum was collected to analyze alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using automatic biochemical instrumentation at Renji Hospital Lab, Shanghai, China.

Histopathological staining and analysis

For HE and Masson staining, 4-μm liver tissue sections were cut from the same position and embedded in paraffin after being stabilized in 10% formalin. Changes in liver tissues were observed under a light microscope.

Evaluation of inflammation activity: scores of inflammation activity were in accordance with chronic liver disease activity[20], and were divided into four parts; i.e., portal area inflammation (P), lobular inflammation (L), patch necrosis (PN) and bridging necrosis (BN, including lobular necrosis). Every item was recorded as 1, 2, 3 or 4 based on degrees of pathological changes. The scores counting formula was: $P + L + 2 \times (PN + BN)$.

Fatty hepatic fibrosis: fibrosis scores were divided into four stages according to the degree of fibrosis in three areas of the liver; i.e., the liver acinus, the portal vein, and bridging fibrosis, as well as the presence or

absence of liver cirrhosis. S1 indicated perisinusoidal space fibrosis of three areas of the local or extensive liver acinus; S2 indicated the above pathological changes with local or extensive periportal fibrosis; S3 indicated S2 pathological changes with local or extensive bridging fibrosis; and S4 indicated fatty liver cirrhosis, forming fibrous septa that divided lobuli hepatic and central veins to the portal area and formed false lobules.

Immunohistochemical analysis

Liver tissue sections (4 μm) were prepared for IL-6 immunohistochemical study. The glass was treated by polylysine to promote cell attachment. Microwave antigen repairing was carried out with 0.01 mol/L citrate buffer solution (pH 6.0). After blocking with rabbit serum, the sections were incubated overnight at 4 ℃ with monoclonal primary antibody against mouse IL-6 (PPMX, Tokyo, Japan). On the next day, liver sections were taken out and washed with PBS three times and were incubated with the second antibody for 1 h at room temperature. Coloration with freshly prepared diaminobenzidine (DAB) was performed, and then the tissues were counterstained with hematoxylin, dewatered, and then mounted with neutral gum. The second antibody in the ElivisionTM plus Polyer HRP (Mouse/Rabbit) IHC Kit and DAB developer were supplied by Manxin Bio Co®, Fuzhou, China. PBS taking the place of the primary antibody was considered as the negative control. We chose 10 views of each section under light microscopy to obtain the average positive absorbance using ImageProplus2.0.

Enzyme linked immunosorbent assay

Liver (1 g) was placed in PBS with 0.1 mmol/L phenylmethyl sulfonylfluoride (PMSF, Sigma) and was then manually homogenized, centrifuged at 100 000 r/min for 15 min at 4 ℃, and the supernatant was removed. Double antibody enzyme linked immunosorbent assay (ELISA) (ELISA kit TNF-α, BD Bioscience, Franklin Lakes, NJ, United States) was used for detection, and the procedure was strictly based on the guidelines provided by the manufacturer.

Western blotting analysis of NF-κB p65, TGF-β1 and α-SMA

Liver tissue was saved in a refrigerator at -80 ℃ after homogenization, and the tissue protein extract solution was prepared by centrifugation. Based on the operating instructions of the bicinchoninic acid protein quantitation kit (Sunbio, Beijing, China), the concentration of protein was detected. After denaturation, each tissue protein was sampled at 50 μg, and reducibility was performed with sodium dodecyl sulfate polyacrylamide gel electrophoresis cataphoresis in an 8% polyacrylamide gel. Sampling after denaturation was performed and electrophoresis was started. The electrophoresis voltage of the condensed glue and separation gel was 80 V and 120 V, respectively. The electrophoresis terminated after bromophenol blue electrophoresis moved to the bottom of the glue, and a damp-dry transmembrane (polyvinylidene fluoride membrane) was applied with a constant 50 mA current for 90 min. The membrane was sealed at room temperature with 5% defatted milk powder prepared with Tris-buffered saline Tween-20 (TBST) and primary antibodies (NF-κB p65, TGF-β1, α-SMA and β-actin, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, United States) and was incubated overnight in a swing bed at 4 ℃. After the film was washed with TBST buffer solution in the swing bed, the second antibody 1:3000 (rabbit polyclonal antibody, Manxin Bio, Fuzhou, China) was incubated at room temperature for 1 h. After being repeatedly washed in TBST buffer solution, DAB staining was performed. After proper staining, the reaction was terminated by water. In a dark room, a nitrocellulose filter was put into a brightening agent with sufficient contact, and was then exposed to light with an X-ray device. The image was developed and fixed. Simultaneous determination of the expression level of β-actin in the same filter was carried out as an internal control. Separate analyses were performed for each sample and the experiment was repeated three times. We obtained the integrated density value by Microsoft BandScan, and the ratio of the target bands to β-actin substantiated the presence of the proteins NF-κB p65, TGF-β1 and α-SMA.

RNA extraction and analysis of mRNA expression of types Ⅰ and Ⅲ collagen, α-SMA and TGF-β1

Total RNA was isolated from snap-frozen liver tissue using Trizol reagent (Invitrogen, Carlsbad, CA, United States) and the ratio between the absorbance values at 260 nm and 280 nm gave an estimate of RNA purity. Real-time (RT)-PCR was performed using a one-step RT-PCR kit from the Shanghai Daweike Biotechnology Company. Two micrograms of the total RNA was chosen and reverse transcription was performed. Its reaction product was placed into a 50-μL PCR reaction system. α-SMA, TGF-β1, types I and Ⅲ collagen, and the specific primer of the internal reference β-actin was used in PCR amplification, and agarose electrophoresis was performed. Electrophoresis results were scanned with a BioSens GelImaging System. For the PCR primer sequences and fragment lengths (Table 1). The real-time survey meter (7500 Sequence Detection System) was obtained from ABI, United States. The PCR conditions were: predegeneration for 2 min at 50 ℃, denaturation for 20 s at 95 ℃, annealing for 45 s at 60 ℃, and extension for 30 s at 72 ℃, with a total of 40 cycles; an internal reference of β-actin was used with predegeneration for 3 min at 94 ℃, denaturation for 20 s at 95 ℃, annealing for 20 s at 60 ℃, extension for 30 s at 72 ℃, with a total of 35 cycles, and extension again for 10 min after the cycles, and then termination at 4 ℃.

Statistical analysis

Data were expressed as mean ± SD. Statistical analysis was performed with a one-way analysis of variance using SPSS17.0 software, followed by Scheffe's test, and com-

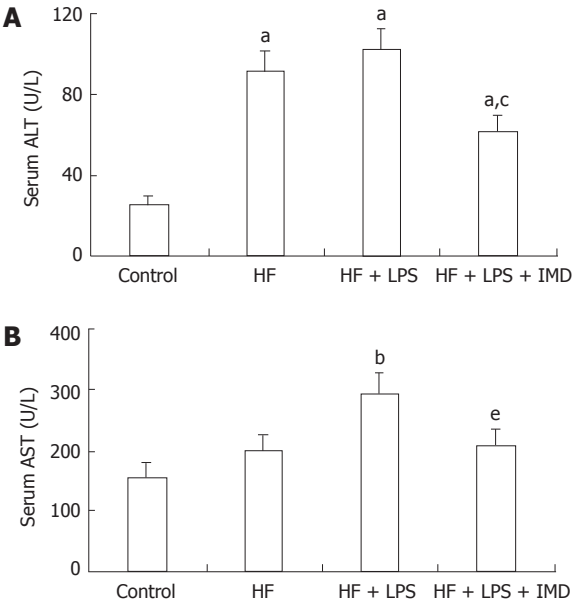

Figure 1 IKK2 inhibitor prevented HF + LPS-induced liver injury, as determined by serum ALT and AST levels. The normal values for ALT and AST were 45 U/L and 160 U/L. Serum ALT (A) and AST (B) were measured in different groups (control group, HF group, LPS-induced HF group and IMD-treated group), data are expressed as mean ± SD. A: $^{a}P < 0.05$ *vs* control group, $^{c}P < 0.05$ *vs* the HF and LPS + HF groups; B: $^{b}P < 0.01$ *vs* the control group, $^{e}P < 0.05$ *vs* the LPS + HF group. HF: High-fat; LPS: Lipopolysaccharide; IMD: IKK2 inhibitor; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

parisons between groups was performed using the Mann-Whitney test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Effects of IKK2 inhibitor (IMD0354) on serum ALT and AST

The levels of ALT and AST for each group are shown in Figure 1. In Figure 1A, the ALT levels of the HF and HF + LPS groups were significantly increased compared to the control group ($P < 0.05$). After treatment with the IKK2 inhibitor (IMD0354), the level of serum ALT in the mice was significantly decreased compared to the HF group ($P < 0.01$), as well as in the HF + LPS group ($P < 0.05$), but was still higher than that of the control group ($P < 0.05$). Figure 1B shows that the change in serum AST was not as significant as that of ALT. The level of serum AST in the HF + LPS group was significantly increased compared to the control group ($P < 0.01$). The level of serum AST when treated with IMD0354 was significantly decreased compared to that of the HF + LPS group ($P < 0.05$).

Effects of IKK2 inhibitor on liver inflammation and fibrosis during liver injury development

HE staining results showed that pathological changes in the mice were in line with the diagnostic gold standard of chronic NASH (Figure 2A). At the same time, typical hepatic fibrosis was observed with Masson staining (Figure 2B). In the control group, the structure of the hepatic lobules was clear without inflammatory cell infiltration in the portal area and without fibrotic tissue hyperplasia. The liver sections of the HF and HF + LPS groups showed that the normal structure of the hepatic lobules was lost, the structure of the blood vessels in the liver was disordered with severe liver cell degeneration, patch necrosis and bridging necrosis, and there were many inflammatory cell infiltrates in the portal area. There was also light to moderate hyperplasia of the broglia fibrils and fibrous septa were formed occasionally. Inflammation and fibrosis scores showed significant differences compared with the control group ($P < 0.05$). In the IMD-treated group, the structure of the hepatic lobules was normal, liver cell degeneration was significantly decreased, and liver cell necrosis and inflammatory cell infiltration were significantly improved. Fibrillar collagen sediment still existed, which was significantly reduced compared with the controls, and its inflammation score was also significantly decreased ($P < 0.05$), but was still higher than that of the control group ($P < 0.05$, Table 1). The results of the fibrosis scores were analyzed by Mann-Whitney statistical methods, and the results showed that there was a significant difference between the HF + LPS + IMD and HF + LPS groups ($Z = -2.35$, $P = 0.018$, $P < 0.05$, Table 1). There were no differences among the other groups.

Table 1 Pathological scores in liver tissues

Groups	$P + L + 2 \times (PN + BN)$	S0	S1	S2	S3	S4	Z
Control	0	5	0	0	0	0	
HF	10.70 ± 2.62^{a}	0	3	2	0	0	
HF + LPS	13.30 ± 3.83^{a}	0	1	3	1	0	
HF + LPS + IMD	$4.60 \pm 3.83^{a,c}$	0	4	1	0	0	-2.35^{e}

The score of inflammation is given by $P + L + 2 \times (PN + BN)$. $^{a}P < 0.01$ *vs* the control group; $^{c}P < 0.01$ *vs* the HF and HF + LPS groups; $^{e}Z = -2.35$, $P = 0.018$, $P < 0.05$ *vs* the HF + LPS group. *P*: Portal area inflammation; L: Lobular inflammation; PN: Patch necrosis; BN: Bridging necrosis; HF: High-fat; LPS: Lipopolysaccharide; IMD: IKK2 inhibitor.

Immunohistochemistry assay for the changes in IL-6 expression in the livers of mice

Previous studies have demonstrated that many cell factors, such as TNF-α and IL-6, play important roles in the NF-κB dependent signaling pathway[21]. Some evidence has indicated that TNF-α, as well as IL-6, participated in the formation of hepatic fibrosis and had a positive correlation with the level of serum hyaluronic acid, laminin type Ⅳ, for example, suggesting that TNF-α, as well as IL-6, not only mediated inflammatory reactions but also participated in the formation of hepatic fibrosis during the promotion of extracellular matrix (ECM) synthesis. In this study, the average absorbance values (*A*) of liver cell positive immunity of mice in each group were analyzed by Image-pro plus 6.0 and statistical analysis was performed, showing that there was a small amount of IL-6 expression in the control and HF groups, and the expression of IL-6 was significantly increased in the HF

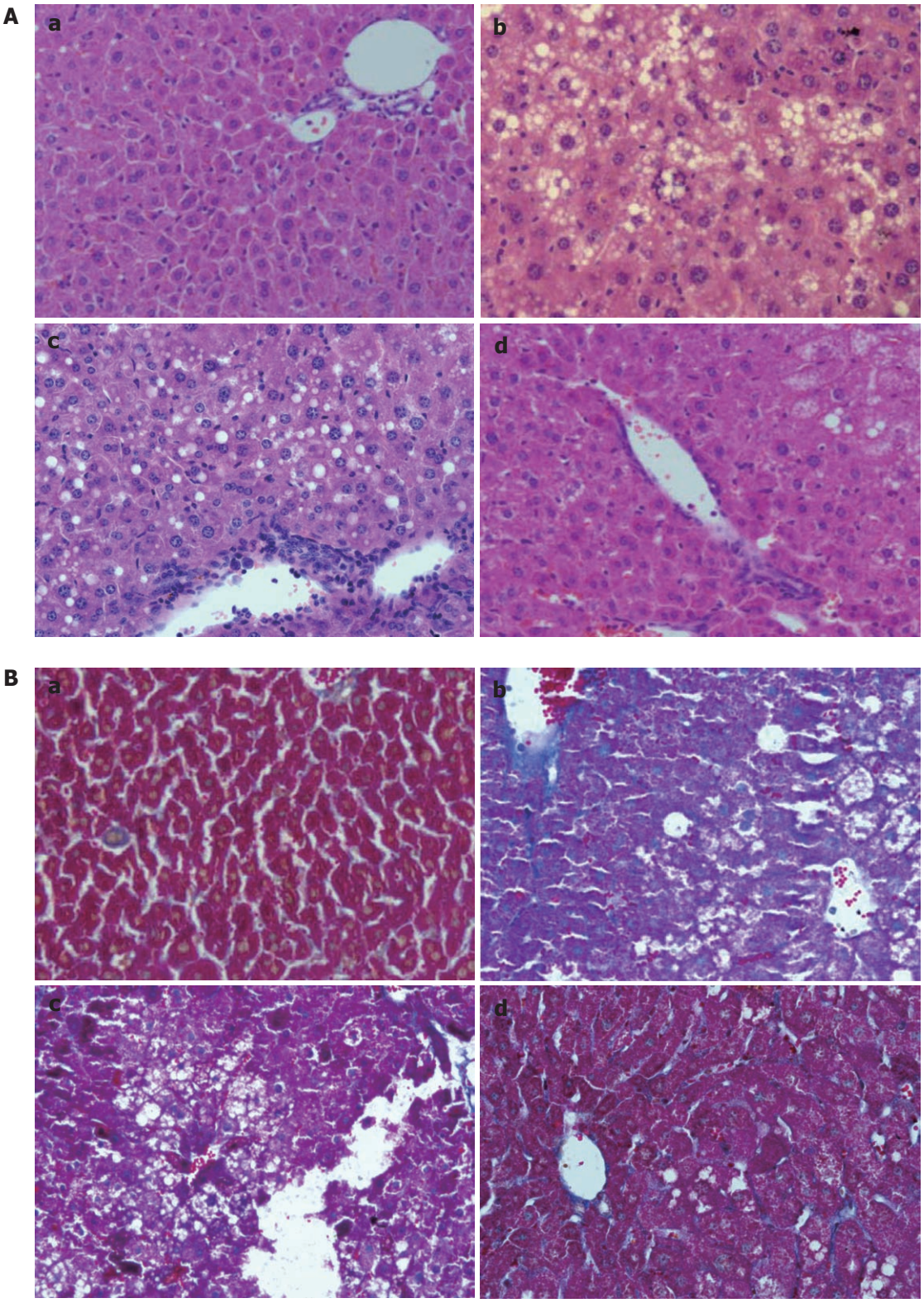

Figure 2 Hematoxylin and eosin stain and Masson staining in sections of (a) control group; (b) HF group; (c) HF + LPS group; and (d) HF + LPS + IMD group. A: Hematoxylin and eosin stain. Macrovesicular steatosis, lobular inflammation and balloon degeneration of hepatocytes were observed in liver sections of HF-treated mice and HF + LPS + treated mice with a significantly large amount of inflammatory cell infiltration surrounding the centrilobular veins of the liver. Significant amelioration was observed in the group treated with IMD (d); B: Masson staining. A thin lining of collagen was observed in the HF group, HF + LPS group and HF + LPS + IMD group. With LPS treatment, there was an increase in the amount of collagen accumulated along the central vein with the presence of collagen in the pericellular area. Treatment with IMD reduced LPS-induced collagen accumulation. HF: High-fat; LPS: Lipopolysaccharide; IMD: IKK2 inhibitor.

group after being activated by LPS ($P = 0.013$, $P < 0.05$). IL-6 expression was mainly concentrated in the liver cell cytoplasm around the central veins and portal area, appearing as brown and grainy, and was also expressed

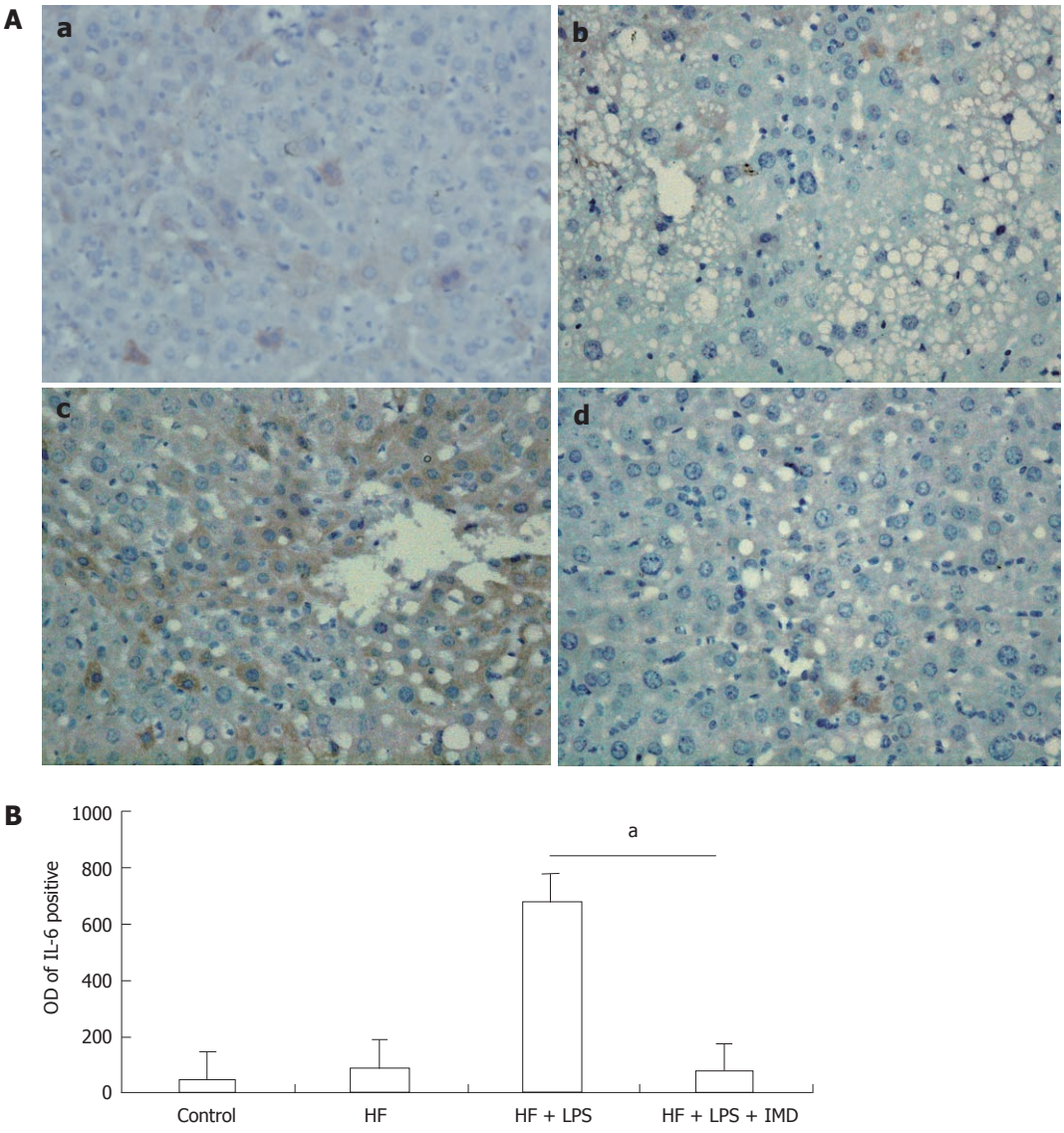

Figure 3 Interleukin-6 expression was assessed by immunohistochemistry. A: Positive staining was observed in hepatocytes in the control group (a), HF group (b), LPS-induced HF group (c) and IMD-treated group (d). B: The optical density (OD) of interleukin-6 (IL-6)-positive areas was measured with ImageProplu6.0 ($^{a}P < 0.05$). HF: High-fat; LPS: Lipopolysaccharide; IMD: IKK2 inhibitor.

in the sinus hepaticus and parts of monocytes. In the HF + LPS group treated with the intervention of IMD, the expression of IL-6 was significantly decreased ($P = 0.012$, $P < 0.05$). There was no significant difference when compared with the HF group ($P = 0.70$, $P > 0.05$), and the expression of IL-6 was higher than the control group (Figure 3).

IKK2 inhibitor (IMD) inhibited an LPS-induced increase in the pro-inflammatory cytokine levels of TNF-α in mice livers

Recent research has shown that the IKK2-NF-κB signal pathway participate in insulin resistance, and cell factors of TNF-α and IL-6 play important roles dependent on NF-κB signals[21], especially in the pathological process of hepatic fibrosis[17,22]. Therefore, TNF-α was the key pro inflammatory factor, which was likely to induce the formation and development of hepatic fibrosis. The protein levels of TNF-α in mouse livers were detected by ELISA. With the development of liver injury, an increased expression of TNF-α was shown in the liver[23]. The levels of TNF-α in mouse livers of the HF and HF + LPS groups were significantly increased compared to the control group ($P < 0.05$). After intervention with the IKK2 inhibitor, the level of TNF-α was significantly reduced compared to the non-intervention group ($P < 0.05$, Figure 4).

IKK2 inhibitor (IMD) decreased nuclear translocation of NF-κB p65 in mice livers in response to LPS and HF exposure

When combined with IκBα in the cytoplasm, NF-κB was inactive. IKK2, as the major subunit of promotion, activated NF-κB and its subunit with phosphorylation, while IMD could inhibit the activation of NF-κB p65 and its nuclear transcription[24]. Therefore, expressions

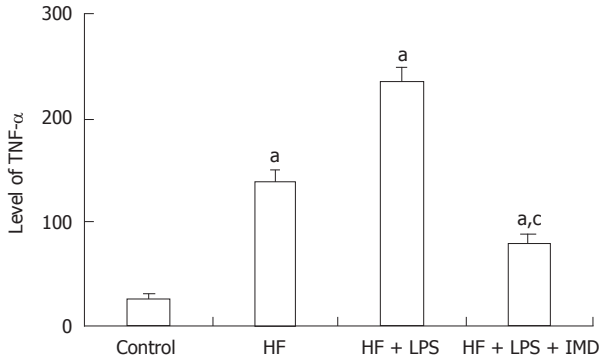

Figure 4 The levels of nuclear factor-κB-dependent pro inflammatory cytokines and tumor necrosis factor-alpha were measured in livers obtained from the control group, HF group, HF + LPS group and HF + LPS + IMD group. $^{a}P < 0.05$, compared with the control group. $^{c}P < 0.05$, significant compared with both the HF and LPS + HF exposed groups. HF: High-fat; LPS: Lipopolysaccharide; IMD: IKK2 inhibitor; TNF-α: Tumor necrosis factor-alpha.

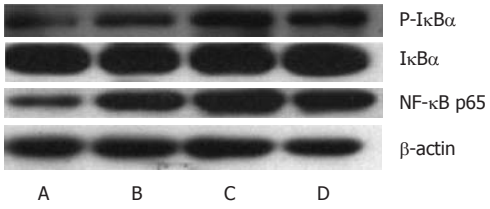

Figure 5 IKK2 inhibitor decreased lipopolysaccharide-induced nuclear translocation of nuclear factor-κB p65 and P-IκBα in livers. Nuclear levels of the p65 subunit of nuclear factor-κB (NF-κB) were measured by Western blotting in different groups (A: Control; B: HF group; C: HF + LPS group; D: HF + LPS + IMD group). β-actin was used as a loading control. Administration of IMD at 30 mg/kg doses decreased the DNA binding activity of NF-κB, which was induced by HF and LPS in mice livers. HF: High-fat; LPS: Lipopolysaccharide; IMD: IKK2 inhibitor.

of NF-κB p65 and P-IκBα in the livers in each group was detected to determine the inhibitory action of IMD. Western blotting showed that the expression of NF-κB p65 in the HF group was increased compared to that of the normal diet group. In the HF group, LPS promoted the expression of NF-κB p65, and P-IκBα increased simultaneously, while intervention by the IKK2 inhibitor reduced the pro-inflammatory role of LPS and significantly reduced the expression of NF-κB p65 and its subunit (Figure 5).

IKK2 inhibitor (IMD) decreased protein levels of TGF-β1 and α-SMA related to fibrosis in livers

TGF-β1 is an important inflammatory factor that stimulates accumulation in the ECM and tissue fibrosis. Our results showed that TGF-β1 expression in the liver was increased under the condition of the HF diet and stimulation of LPS. While α-SMA was a marker of HSC activation, its variation tendency was similar to TGF-β1. In the mouse liver model group, the expression of α-SMA was significantly increased compared to the control group. After application of the IKK2 inhibitor, the protein expression of TGF-β1 in livers decreased, and the expression of α-SMA was reduced accordingly.

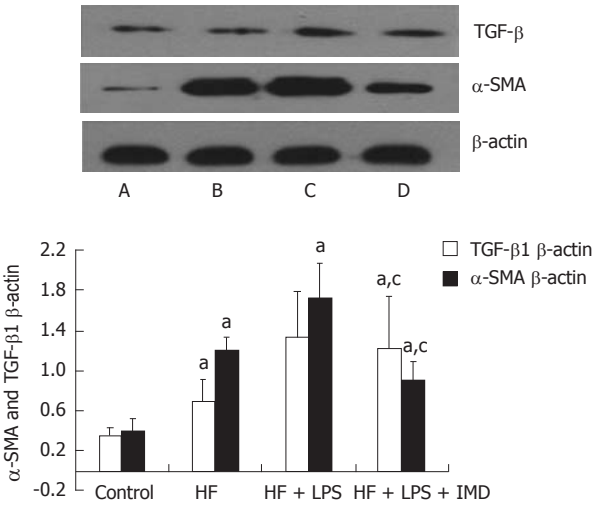

Figure 6 Western blotting analysis of tumor growth factor-beta1, alpha-smooth muscle actin proteins were measured that were involved in IKK2-nuclear factor-κB pathways in the liver in different groups (A: Control; B: HF group; C: HF + LPS group; D: HF + LPS + IMD group). β-actin was used as a loading control. The levels of tumor growth factor-beta1 (TGF-β1) and alpha-smooth muscle actin (α-SMA) measured in livers were increased in the HF and HF + LPS groups. IKK2 inhibitor significantly inhibited LPS and HF-induced expression of TGF-β1 and α-SMA in mouse livers. The ratio of TGF-β1 and α-SMA/β-actin in the liver was increased in other groups, compared with the control group, $^{a}P < 0.05$. IKK2 inhibitor normalized TGF-β1 and α-SMA significantly compared with the HF or LPS + HF groups. $^{c}P < 0.05$. HF: High-fat; LPS: Lipopolysaccharide; IMD: IKK2 inhibitor.

Table 2 Oligonucleotide sequences used in real-time polymerase chain reaction

mRNA	Sequence	Length (bp)
Type Ⅰ (Ⅰ α) collagen	F: ACAGTGGTGAACCTGGTGCT	151
	R: CTCCTTTGGCACCAGTGTCT	
Type Ⅲ collagen	F: GGAGCCCCTGGACTAATAG	193
	R: ATCCATCTTTGCCATCTTCG	
α–SMA	F: TGCTGTCCCTCTATGCCTCT	185
	R: GAAGGAATAGCCACGTCAG	
TGF-β1	F: CTTGCCCTCTACAACCAACA	189
	R: CTTGCGACCCACGTAGTAGA	
β-actin	F: TGTGTCCGTCGTGGATCTGA	126
	R: CTTGCGACCCACGTAGTAGA	

α–SMA: Alpha-smooth muscle actin; TGF-β1: Tumor growth factor-beta1.

Correspondingly, activation of HSCs and the formation of collagen decreased, leading to effectively preventing hepatic fibrosis (Figure 6).

IKK2 inhibitor IMD inhibited α-SMA, TGF-β1, types Ⅰ (α Ⅰ) and Ⅲ collagen and mRNA expression in LPS-stimulated mice

The formula described previously was used to measure mRNA relative expression (amount = $2^{-\Delta ct} \times 100\%$), and the relative expression levels of α-SMA, type Ⅰ (α Ⅰ) collagen, type Ⅲ collagen and TGF-β1 mRNA were obtained. RT-PCR was performed with the mouse primers shown in Table 2. The results showed that the expression of TGF-β1 mRNA in the HF group and the HF + LPS group was higher than the control group ($P < 0.05$),

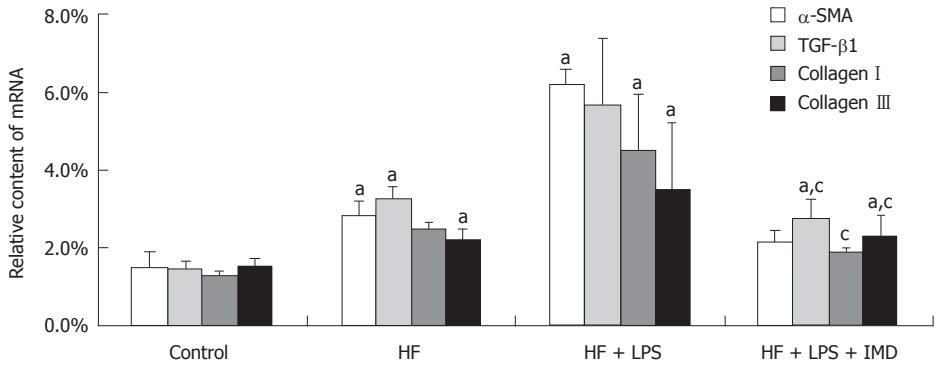

Figure 7 The IKK2 inhibitor inhibited LPS and HF-induced increases in pro inflammatory cytokine levels in mouse livers. The level of tumor growth factor-beta1 (TGF-β1) was measured in the livers of mice in the control, HF, HF + LPS and HF + LPS + IMD groups. Also, expression of the fibrosis index, such as alpha-smooth muscle actin (α-SMA), type Ⅰ collagen and type Ⅲ collagen, were detected in the four groups by real-time polymerase chain reaction. The level of TGF-β1 measured in livers was increased in the HF and HF + LPS groups, compared with the control group, $^{a}P < 0.05$. The mRNA content of type Ⅰ collagen in the HF group and type Ⅲ collagen in the HF + LPS group were significantly higher, $^{a}P < 0.05$, compared with the control group. Intraperitoneally administered IKK2 inhibitor normalized the expression of TGF-β1, as well as the contents of α-SMA, type Ⅰ and type Ⅲ collagen mRNA, compared with the HF or LPS + HF groups. $^{a}P < 0.05$, compared with the control group. $^{c}P < 0.05$, compared with both HF group and LPS + HF exposed group. **HF: High-fat; LPS: Lipopolysaccharide; IMD: IKK2 inhibitor.**

which was significantly decreased after intervention of IMD. In addition, the fibrosis indexes of α-SMA, type Ⅰ collagen and type Ⅲ collagen in the model group were also increased. In addition, the contents of type Ⅰ collagen in the HF diet group and type Ⅲ collagen in the HF + LPS group were significantly increased, independently, compared to the control group ($P < 0.01$, $P < 0.05$, respectively). After intervention with IMD, the levels of α-SMA, type Ⅰ (α Ⅰ) collagen and Ⅲ collagen were significantly decreased compared to the HF + LPS group ($P < 0.05$). However, there was no significant difference in α-SMA and type Ⅲ collagen with intervention of IMD and the normal group ($P > 0.05$, Figure 7).

DISCUSSION

NAFLD includes simple liver steatosis, NASH and liver cirrhosis, while NASH has become a central issue of chronic liver disease with worldwide attention[7]. Currently, the pathogenesis of NAFLD is not clear, and the hypothesis of "secondary strike" has been widely accepted. It is well known that insulin resistance is involved in the process, and inflammatory reaction, lipid peroxidation, and oxidative stress also play important roles[25-27]. The best measure for preventing the progression of hepatic fibrosis is to prevent or reverse the initial cascade reactions of fibrosis[28]. Therefore, inhibition of the generation of inflammatory factors effectively reduces HSC activation, decreases accumulation in the ECM, and fundamentally reverses fibrosis[29]. The expression of NF-κB is significantly increased in NASH patients, and TNF-α, IL-6, TGF-β, and other inflammatory factors also showed high expression levels[23]. Further research has found that activation of NF-κB is the key step in regulating gene expression of various kinds of pro inflammatory factors in NASH patients[23]. TNF-α is the key pro inflammatory factor and it induces the formation and development of hepatic fibrosis. TGF-β1 mediates the synthesis of different kinds of collagen with time dependence. However, inhibition of TGF-β1 significantly reduces the synthesis of collagen and sedimentation of the ECM[30].

It has been reported that blockage of IKKβ (IKK2) significantly reduces the incidence rate of liver steatosis and improves NASH pathologically[31]. Is it possible that IKKβ (IKK2)-NF-κB is also a key in improving and even reversing hepatic fibrosis? Various macromolecular protein-joining enzymes, including IKKβ (IKK2), IKK or NF-κB inhibitors, have become new types of anti-inflammatory agents; therefore, many researchers have tried to inhibit NF-κB-mediated proinflammatory responses based on these agents. The IKK2 inhibitor played a specific anti-inflammatory role through inhibition of the major subunit IKKβ, which served as a promotor in the IKK protein kinase complex center. In our study, the IKK2 inhibitor (IMD 0354) was used in liver injury in mice. We detected its inhibitory effect on liver NF-κB-dependent inflammatory factors, changes in liver function, histological changes and, at the same time, the expression of TGF-β1 involving fibrosis and relevant fibrosis indexes, such as α-SMA, type Ⅰ collagen and type Ⅲ collagen. It is warranted to investigate the potential therapeutic effect of IMD on hepatic fibrosis.

In our study, during HF-diet-induced chronic non-alcoholic hepatic injury in mice, the serology index of ALT was doubly higher than that of the control group. As for pathological changes, moderate to severe steatosis was observed, inflammatory infiltration was found in the lobules, local inflammatory infiltration was detected in the portal area, Masson staining showed fibrous tissue hyperplasia, Western blotting and RT-PCR results demonstrated that TGF-β1 expression increased, α-SMA content was raised, and there was sedimentation of types Ⅰ and Ⅲ collagen. Therefore, a hepatic injury model with typical inflammation and fibrosis pathological manifestation was successfully established by an HF diet and intraperitoneal injection of LPS, stimulating activation of NF-κB and promoting an inflammatory reaction[32-34].

When chronic hepatic injury occurs, different initial causative agents trigger the activation of HSCs, activation of the Janus kinase-signal transducers and activators of transcription signal transduction pathway[35], promotion of α-SMA expression in sinus hepaticus cells, further proliferation and activation, and synthesis and secretion of the ECM and collagen, which finally enhances the occurrence of hepatic fibrosis. Thus, expression of α-SMA has been considered as one of the dominant features of HSC activation, and has become an important evaluation index for hepatic fibrosis. In our study, when the mice were stimulated with LPS and an HF diet, the expression of α-SMA increased in the liver. At the same time, sedimentation of types I and Ⅲ collagen also occurred, suggesting that HSC was triggered and activated, and then started the process of hepatic fibrosis. However, the results for the group treated with IMD showed that expression of α-SMA, as well as sedimentation of types I and Ⅲ collagen, were significantly reduced, suggesting that inhibition of inflammatory factor expression also effectively suppressed HSC activation, and accordingly blocked the occurrence of hepatic fibrosis from the source.

Fatty tissue was the principle source of cell factors, liver steatosis promoted macrophage infiltration, and activation of HSC promoted the cascading release of many kinds of cell factors with an intensive pro inflammatory role, which further made the pathological changes and insulin resistance more serious[36,37]. We found that inflammatory cell and inflammatory factor expression after stimulation with LPS significantly increased compared to that in the HF group. Liver steatosis in the mice treated with intraperitoneal injection of the IKK2 inhibitor was significantly improved, and inflammatory factors released from the hepatic cell fat were correspondingly reduced compared to that of the HF and LPS groups. In addition, it was shown that the IKK2 inhibitor could significantly reduce pro inflammatory stimulation by LPS, and even reverse the fibrosis process in a mouse hepatic injury model. Western blots demonstrated that NF-κB p65 activation was significantly inhibited, and NF-κB-dependent pro inflammatory factors, such as IL-6, were simultaneously suppressed. Separation of NF-κB and its suppressor factor IκBα resulted in continuous activation of intercellular adhesion molecule-1 and other cell factors, and finally the up regulation of IL-6. The increase in IL-6 stimulated HSC proliferation, induced production of multiple acute-phase proteins, and promoted ECM sedimentation by facilitating matrix degeneration or interaction with its adhesion receptor, leading to significant hepatic fibrosis[38,39]. Similarly, the high expression of IL-6 significantly promoted liver apoptosis. In our study, it was found that after intervention with the IKK2 inhibitor, NF-κB p65 activation was significantly inhibited, IL-6 expression in the livers of LPS model mice was significantly decreased with the improvement of serology indexes and histological changes, and blocking IL-6 expression significantly improved hepatic injury, which could demonstrate that the IKK2 inhibitor could improve hepatic fibrosis by inhibition of the inflammatory factor IL-6.

It is known that TNF-α promotes insulin resistance and the development of liver inflammation, which is related to multiple cell factors, and induces the synthesis of IL-1, IL-6 and C-reactive protein, including caspase 3 and growth arrest and DNA-damage-inducible beta. Adiponectin inhibits expression of TNF-α, as well as other inflammatory factors, with positive feedback[40,41]. A peroxisome proliferator-activated receptor antagonist blocked TNF-α mediated insulin resistance, and it also had intensive anti-inflammatory action[42,43], which made the inflammation signal transduction pathway become a multiple cross and participated in the process of hepatic fibrosis[44]. The level of TNF-α could better reflect the regulatory condition of the inflammatory reaction in hepatic fibrosis. Hepatocellular carcinoma (HCC) invariably develops within a setting of chronic inflammation caused by metabolic liver disease or autoimmunity. Mechanisms that link these two processes are not completely understood, but transcription factors of the NF-κB family have been suggested to be involved. Cytokines such as IL-6 are clearly pivotal players, and high levels of serum IL-6 correlate positively with tumor size and with poor prognosis in HCC patients[45]. Our results showed that the levels of IL-6 and TNF-α in mouse livers in which hepatic fibrosis existed increased, while the IKK2 inhibitor reversed such an imbalance, and α-SMA expression in the liver and sedimentation of collagen I and collagen Ⅲ decreased, illustrating that TNF-α improved liver pathological changes in hepatic fibrosis in mice, relieved inflammatory cell infiltration, and reduced fiber hyperplasia by the IKK2-NF-κB-dependent signaling pathway.

During the process of HSCs activation, TGF-β1 plays a major role as a fibroblast growth factor[46], and the major stimulating factor promoting HSCs to accumulate ECM[47]. TGF-β1 receptors on the surface of HSC complete the signaling pathway combined with Smads[48], which continuously stimulates HSC activation, and finally transcribes target gene expression in the HSC nucleolus, mainly including type I collagen, which plays a key regulatory role in ECM metabolism and function. However, there is evidence demonstrating that NF-κB does not directly activate HSCs[39]. In our experiment, when injury and hepatic fibrosis occurred, protein and gene expression of TGF-β1 increased. While inhibiting the activation of NF-κB certainly reduced the activation of HSCs, TGF-β1 expression was decreased, and the expressions of various kinds of fibrosis factors, such as α-SMA and type I and type Ⅲ collagen, decreased. Therefore, we suggest that indirect correlation or other pathways exist between NF-κB and HSCs, which indirectly reduce TGF-β1 expression, decrease continuous activation of HSCs, and then improve the fibrosis process.

In brief, our *in vivo* experiment demonstrated that an IKK2 inhibitor could significantly decrease the expres-

sion of various inflammatory factors in mouse livers after exposure to LPS, which could play an anti-fibrosis role in inhibiting inflammation, reducing collagen content in liver tissues, and decreasing the expression of hepatic fibrosis correlation factors. IMD inhibited the phosphorylation activation of IκBα and NF-κB stimulated by LPS. Meanwhile, the expression of NF-κB-dependent inflammatory cytokines were suppressed, illustrating that inflammatory factors inducing hepatic injury were effectively reduced by inhibition of the NF-κB signaling pathway. In addition, we found that various fibrosis markers, such as TGF-β1 and α-SMA, as well as type I collagen and type Ⅲ collagen, were decreased in the liver, demonstrating that HSC activation was decreased and ECM accumulation was reduced. Therefore, it was presumed that the mechanism of the IKK2 inhibitor in anti hepatic fibrosis might be relevant, with the inhibition of cell factors promoting HSC activation, indirect inhibition of HSC proliferation, suppression of continuous activation of HSCs by TGF-β1, and then secretory volume and expression levels of α-SMA being reduced. Finally, the extent of hepatic fibrosis was decreased. The major pathological changes of chronic fatty liver disease are insulin resistance and inflammatory reactions[49,50]. In summary, the NF-κB signaling pathway participates with the IKK2 inhibitor playing an influential role in NASH and hepatic fibrosis. Inhibiting the production of a variety of inflammatory factors could effectively reduce HSC activation, decrease accumulation in the ECM, and then reduce fibrosis formation.

COMMENTS

Background

The nuclear factor-κB (NF-κB) signaling pathway improves insulin resistance and fat accumulation in the development of nonalcoholic fatty liver disease (NAFLD). Inhibition of NF-κB activation by an IKK2 inhibitor could effectively suppress the expression of many kinds of inflammatory factors, and even improve hepatic fibrosis. Therefore, the authors of this study investigated the potential therapeutic prospects of the IKK2-NF-κB signaling pathway in the reversion of fibrosis in NAFLD.

Research frontiers

The study is believed to be the first to evaluate the role of IKK2-NF-κB signals in NAFLD. The potential effect of an IKK2 inhibitor is likely to block inflammation through inhibiting NF-κB activation. The IKK2 inhibitor may play an important role in the occurrence and development of NAFLD.

Innovations and breakthroughs

This study explored one of the possible mechanisms of inflammation, which could produce a potentially facilitative effect in the occurrence and development of NAFLD.

Applications

This study provides an experimental basis for future studies on the role of IKK2 in NAFLD. Control of the expression level of IKK2 in the liver may become a new possibility for therapy of NAFLD.

Terminology

In the present study, the authors tested the effect of IKK2 in NAFLD in mice, and found a facilitative effect on the occurrence and development of NAFLD.

Peer review

The paper should be accepted with some minor revisions. IκB kinase-beta inhibitor attenuates hepatic fibrosis in mice liver injury and its potential mechanisms.

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BRIEF ARTICLE

Meta-analysis of robotic and laparoscopic surgery for treatment of rectal cancer

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Abstract

AIM: To conduct a meta-analysis to determine the relative merits of robotic surgery (RS) and laparoscopic surgery (LS) for rectal cancer.

METHODS: A literature search was performed to identify comparative studies reporting perioperative outcomes for RS and LS for rectal cancer. Pooled odds ratios and weighted mean differences (WMDs) with 95% confidence intervals (95% CIs) were calculated using either the fixed effects model or random effects model.

RESULTS: Eight studies matched the selection criteria and reported on 661 subjects, of whom 268 underwent RS and 393 underwent LS for rectal cancer. Compared the perioperative outcomes of RS with LS, reports of RS indicated favorable outcomes considering conversion (WMD: 0.25; 95% CI: 0.11-0.58; P = 0.001). Meanwhile, operative time (WMD: 27.92, 95% CI: -13.43 to 69.27; P = 0.19); blood loss (WMD: -32.35, 95% CI: -86.19 to 21.50; P = 0.24); days to passing flatus (WMD: -0.18, 95% CI: -0.96 to 0.60; P = 0.65); length of stay (WMD: -0.04; 95% CI: -2.28 to 2.20; P = 0.97); complications (WMD: 1.05; 95% CI: 0.71-1.55; P = 0.82) and pathological details, including lymph nodes harvested (WMD: 0.41, 95% CI: -0.67 to 1.50; P = 0.46), distal resection margin (WMD: -0.35, 95% CI: -1.27 to 0.58; P = 0.46), and positive circumferential resection margin (WMD: 0.54, 95% CI: 0.12-2.39; P = 0.42) were similar between RS and LS.

CONCLUSION: RS for rectal cancer is superior to LS in terms of conversion. RS may be an alternative treatment for rectal cancer. Further studies are required.

Key words: Robotic surgery; Laparoscopic surgery; Rectal cancer; Da Vinci robotic system; Meta-analysis

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INTRODUCTION

Over the past 30 years, laparoscopic surgery (LS) has revolutionized general surgical practice, above all affecting

surgery of the gastrointestinal (GI) tract[1,2]. However, with regard to rectal cancer, there are several technical drawbacks to LS, including limited range of motion of instruments in a narrow pelvic cavity, related loss of dexterity, and an inadequate visual field associated with unstable camera view and assistant's traction, which are not under the surgeon's control[3]. Technical advantages of the da Vinci robotic system could overcome the limitations of LS for rectal cancer, by giving the surgeon a 3D view, better ergonomics, enhanced dexterity, precision and control due to the 3D optical system and EndoWrist® Instruments.

Although surgical robots have been successfully applied to a number of disciplines, most notably urological and cardiac procedures[4,5], robotic rectal surgery remains in its infancy. Most studies have been limited by small sample size and a single institution design. To overcome these limitations, a meta-analysis of studies comparing robotic surgery (RS) and LS for rectal cancer should be performed. The aim of this meta-analysis was to determine the relative merits of RS and LS for rectal cancer.

MATERIALS AND METHODS

Study selection

The Pubmed, Embase, Cochrane Library, Ovid, and Web of Science databases were searched systematically for all articles published before June 2011 to compare RS and LS for rectal cancer. The terms used for the search were: "robotic" and "rectal cancer". Only studies in the English language were considered for inclusion. Reference lists of all retrieved articles were manually searched for additional studies. Two reviewers independently extracted the data from each study. All relevant text, tables and figures were reviewed for data extraction. Discrepancies between the two reviewers were resolved by discussion and consensus.

Criteria for inclusion and exclusion

For inclusion in the meta-analysis, a study had to fulfill the following criteria: (1) compare the outcomes of RS and LS, regardless of other diseases; (2) report on at least one of the outcome measures mentioned below; and (3) if dual (or multiple) studies were reported by the same institution and/or authors, either the one of higher quality or the most recent publication was included in the analysis.

Abstracts, letters, editorials and expert opinions, reviews without original data, case reports and studies lacking control groups were excluded. The following studies or data were also excluded: (1) the outcomes and parameters of patients were not clearly reported (e.g., with no clearly reported outcomes of SD; (2) it was impossible to extract the appropriate data from the published results; and (3) there was overlap between authors or centers in the published literature.

Outcomes of interest

The following outcomes were used to compare the two operative techniques: (1) intraoperative data, which included operative time, blood loss and conversion; (2) postoperative data, which included complication, days to passing flatus, and length of stay; and (3) pathological details, which included lymph nodes harvested, distal resection margin (DRM), and positive circumferential resection margin (PCRM) which was defined as a circumferential resection margin (CRM) of $\leqslant$ 1 mm.

Data extraction

Two reviewers independently extracted the following parameters from each study: (1) first author and year of publication; (2) study population characteristics; (3) number of subjects operated on with each technique; and (4) intraoperative data, postoperative data, and pathological details.

Statistical analysis

The meta-analysis was performed using the Review Manager (RevMan) software, version 4.2.2. We analyzed dichotomous variables using estimation of odds ratios with a 95% confidence interval (95% CI) and continuous variables using weighted mean difference (WMD) with a 95% CI. Pooled effect was calculated using either the fixed effects model or random effects model. Heterogeneity was evaluated by χ^2 and I^2. We considered heterogeneity to be present if the I^2 statistic was > 50%. $P < 0.05$ was considered significant.

RESULTS

Selection of trials

The initial search strategy retrieved 154 publications, after screening all titles, abstracts, and full-text. A total of eight studies met our entry criteria and were retrieved for more detailed evaluation. The characteristics of these eight studies are summarized in Table 1[6-13]. Eight studies [six non-randomized controlled trials (NRCTs), two randomized controlled trials (RCTs)] included a total of 661 patients: 268 in the RS group and 393 in the LS group. Two studies were conducted in United States[7,13], three in Korea[6,8,12], two in Italy[10,11], and one in Romania[9]. The sample size of each study varied from six to 123 patients. In the included studies, six were considered as level of evidence 3, and the remaining 2 as level of evidence 2 (according to the grading of the Centre of Evidence-Based Medicine, Oxford, United Kingdom; http://www.cebm.net/index.aspx?o=5653).

In these studies, patients in the two groups were matched for operation time[6,9,11,12], blood loss[9,11], conversion[6-13], complications[6-13], days to passing flatus[6,12], length of stay[6,11,12], lymph nodes harvested[6,9,11,12], DRM[6,11,12], and PCRM[6,7,10].

Meta-analysis of intraoperative data

In four studies, operative time showed that there was no significant difference between the two groups. Analysis of the pooled data revealed that the two groups did not differ significantly in this regard (WMD: 27.92, 95% CI:

Table 1 Characteristics of included studies

Study	Country	Group	No. of patients	Mean age (yr)	Gender (M/F)	Level of evidence
Park	Korea	RS	52	57.3 (± 12.3)	28/24	3
et al[6]		LS	123	65.1 (± 10.3)	70/53	
Baek	United States	RS	41	63.6 (48-87)	25/16	3
et al[7]		LS	41	63.7 (42-88)	25/16	
Kwak	Korea	RS	59	60 (53-68)	39/20	3
et al[8]		LS	59	59 (53-69)	42/17	
Popescu	Romania	RS	38	53 (± 11.27)	23/15	3
et al[9]		LS	84	60 (± 12.27)	51/33	
Bianchi	Italy	RS	25	69 (33-83)	18/7	2
et al[10]		LS	25	62 (42-77)	17/8	
Patriti	Italy	RS	29	68 ± 10	NA	3
et al[11]		LS	37	69 ± 10	NA	
Baik	Korea	RS	18	57.3 ± 6.3	14/4	2
et al[12]		LS	18	62.0 ± 9.0	14/4	
Pigazzi	United States	RS	6	60 (42-78)	4/2	3
et al[13]		LS	6	70 (57-88)	2/4	

NA: Not available; RS: Robotic surgery; LS: Laparoscopic surgery.

-13.43 to 69.27; $P = 0.19$) (Figure 1A).

In two studies, blood loss did not differ significantly between the two groups. Analysis of the pooled data revealed that the two groups did not differ significantly in this regard (WMD: -32.35, 95% CI: -86.19 to 21.50; $P = 0.24$) (Figure 1B).

In all eight studies, conversion was found to be significantly lower in the RS group than in the LS group. Moreover, analysis of the pooled data revealed that conversion for RS was significantly lower by 0.25-fold (WMD: 0.25; 95% CI: 0.11-0.58; $P = 0.001$) (Figure 1C).

Meta-analysis of postoperative outcomes

In two studies, number of days to passing flatus was significantly lower in the RS group *vs* the LS group. Meanwhile, analysis of the pooled data revealed that the two groups did not differ significantly in this regard (WMD: -0.18, 95% CI: -0.96 to 0.60; $P = 0.65$) (Figure 1D).

In three studies, length of stay was found to be no different in the RS group and the LS group. Meanwhile, analysis of the pooled data revealed that the two groups did not differ significantly in this regard (WMD: -0.04; 95% CI: -2.28 to 2.20; $P = 0.97$) (Figure 1E).

In all eight studies, complications were found to be no different in the RS group and the LS group. Meanwhile, analysis of the pooled data revealed that the two groups did not differ significantly in this regard (WMD: 1.05; 95% CI: 0.71-1.55; $P = 0.82$) (Figure 1F).

Meta-analysis of pathological details

In the four studies, lymph nodes harvested showed that there was no significant difference between the two groups. Analysis of the pooled data revealed that the two groups did not differ significantly in this regard (WMD: 0.41, 95% CI: -0.67 to 1.50; $P = 0.46$) (Figure 1G).

In three studies, DRM was found to be significantly lower in the RS group than the LS group. Meanwhile, analysis of the pooled data revealed that the two groups did not differ significantly in this regard (WMD: -0.35, 95% CI: -1.27 to 0.58; $P = 0.46$) (Figure 1H).

In three studies, PCRM showed that there was no significant difference between the two groups. Analysis of the pooled data revealed that the two groups did not differ significantly in this regard (WMD: 0.54, 95% CI: 0.12-2.39; $P = 0.42$) (Figure 1I).

Heterogeneity

A significant heterogeneity was recognized in the following two factors: operative time, blood loss, days to passing flatus, length of stay and DRM.

DISCUSSION

Meta-analysis could be used to evaluate the existing literature in both qualitative and quantitative ways by comparing and integrating the results of different studies and taking into account variations in characteristics that could influence the overall estimate of the outcome of interest[14]. Although meta-analysis has traditionally been applied and best confined to RCTs, meta-analytical techniques using NRCTs might be a good method in some clinical settings in which either the number or the sample size of RCTs was insufficient[15]. To the best of our knowledge, this was the first comprehensive meta-analysis comparing RS versus LS for rectal cancer.

RS is often perceived as being more time-consuming, because of the additional set-up time required[16]. It usually requires two steps for rectal cancer[17,18]. After dissection of the left colon and sigmoid colon and division and ligation of the inferior mesenteric vessels, the da Vinci system must be moved for the next step. However, moving the da Vinci system is a time-consuming and difficult procedure because the robotic devices are heavy and bulky. This meta-analysis revealed that there was no significant difference in operative time between RS and LS. This finding could be attributable to the shortened learning curve, and it has been suggested that the intuitive controls of robotic systems, more comparable with open surgery, could shorten the learning curve, even in the hands of relatively inexperienced laparoscopic surgeons[19]. As we overcame the learning curve with experience and prevented collisions by properly positioning the robotic ports, the operation time decreased. There was no significant difference in blood loss when comparing RS and LS.

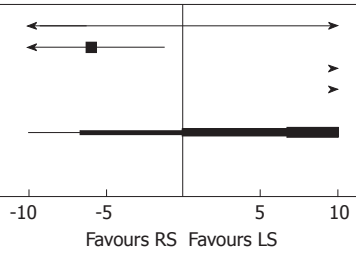
Conversion to open surgery and complications are critical in minimally invasive rectal cancer surgery, because converted patients have higher complication rates[20] and, in one series at least, worse oncological outcomes[21]. Conversion rate was significantly lower in the RS group than in the LS group. Lower conversion with RS might have been due to superior exposure and visualization of the operating field in the pelvis, thanks to the ability of the fixed fourth arm to grip and maneuver organs; the ability of the surgeon to move the 3D camera as required; and the greater ease of dissection afforded by the

A

Review: Meta-analysis of robotic and laparoscopic surgery for treatment of rectal cancer
Comparison: 01 Intraoperative data
Outcome: 01 Operative time

Study or sub-category	*N*	RS mean (SD)	*N*	LS mean (SD)	WMD (random) 95% CI	Weight %	WMD (random) 95% CI
Baik *et al*[12] 2008	18	217.10 (51.60)	18	204.30 (51.90)		22.58	12.80 (-21.01, 46.61)
Patriti *et al*[11] 2009	29	202.00 (12.00)	37	208.00 (7.00)		26.49	-6.00 (-10.92, -1.08)
Popescu *et al*[9] 2010	38	212.00 (47.23)	84	182.00 (37.23)		25.45	30.00 (13.00, 47.00)
Park *et al*[6] 2011	52	232.60 (52.40)	123	158.10 (49.20)		25.49	74.50 (57.81, 91.19)
Total (95% CI)	137		262			100	27.92 (-13.43, 69.27)

Test for heterogeneity: χ^2 = 93.41, df = 3 (P < 0.00001), I^2 = 96.8%
Test for overall effect: Z = 1.32 (P = 0.19)

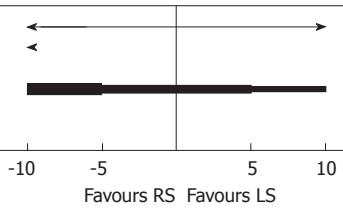


B

Review: Meta-analysis of robotic and laparoscopic surgery for treatment of rectal cancer
Comparison: 01 Intraoperative data
Outcome: 02 Blood loss

Study or sub-category	*N*	RS mean (SD)	*N*	LS mean (SD)	WMD (random) 95% CI	Weight %	WMD (random) 95% CI
Patriti *et al*[11] 2009	29	137.40 (156.00)	37	127.00 (169.00)		29.23	10.40 (-68.27, 89.07)
Popescu *et al*[9] 2010	38	100.00 (50.00)	84	150.00 (50.00)		70.77	-50.00 (-69.16, -30.84)
Total (95% CI)	67		121			100	-32.35 (-86.19, 21.50)

Test for heterogeneity: χ^2 = 2.14, df = 1 (P = 0.14), I^2 = 53.2%
Test for overall effect: Z = 1.18 (P = 0.24)

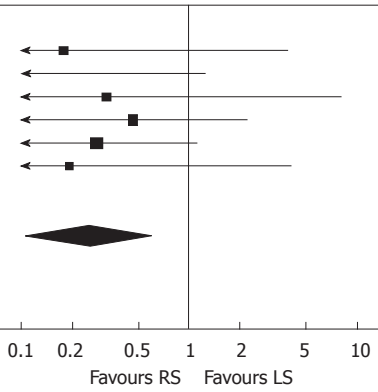


C

Review: Meta-analysis of robotic and laparoscopic surgery for treatment of rectal cancer
Comparison: 01 Intraoperative data
Outcome: 03 Conversion

Study or sub-category	RS *n/N*	LS *n/N*	OR (fixed) 95% CI	Weight %	OR (fixed) 95% CI
Pigazzi *et al*[13] 2006	0/6	0/6			Not estimable
Baik *et al*[12] 2008	0/18	2/18		9.17	0.18 (0.01, 3.99)
Patriti *et al*[11] 2009	0/29	7/37		24.51	0.07 (0.00, 1.26)
Bianchi *et al*[10] 2010	0/25	1/25		5.54	0.32 (0.01, 8.25)
Popescu *et al*[9] 2010	2/38	9/84		20.01	0.46 (0.10, 2.25)
Baek *et al*[7] 2011	3/41	9/41		31.42	0.28 (0.07, 1.13)
Kwak *et al*[8] 2011	0/59	2/59		9.34	0.19 (0.01, 4.11)
Park *et al*[6] 2011	0/52	0/123			Not estimable
Total (95% CI)				100	0.25 (0.11, 0.58)

Total events: 5 (RS), 30 (LS)
Test for heterogeneity: χ^2 = 1.46, df = 5 (P = 0.92), I^2 = 0%
Test for overall effect: Z = 3.20 (P = 0.001)

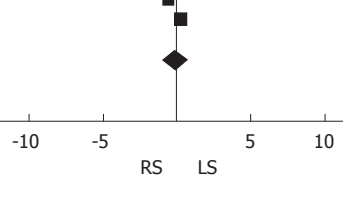


D

Review: Meta-analysis of robotic and laparoscopic surgery for treatment of rectal cancer
Comparison: 02 Postoperative data
Outcome: 01 Days to passing flatus

Study or sub-category	*N*	RS mean (SD)	*N*	LS mean (SD)	WMD(random) 95% CI	Weight %	WMD (random) 95% CI
Baik *et al*[12] 2008	18	1.80 (0.40)	18	2.40 (1.30)		47.61	-0.60 (-1.23, 0.03)
Park *et al*[6] 2011	52	3.20 (1.80)	123	3.00 (1.10)		52.39	0.20 (-0.33, 0.73)
Total (95% CI)	70		141			100	-0.18 (-0.96, 0.60)

Test for heterogeneity: χ^2 = 3.66, df = 1 (P = 0.06), I^2 = 72.7%
Test for overall effect: Z = 0.45 (P = 0.65)



E

Review: Meta-analysis of robotic and laparoscopic surgery for treatment of rectal cancer
Comparison: 02 Postoperative data
Outcome: 02 Length of stay

Study or sub-category	*N*	RS mean (SD)	*N*	LS mean (SD)	WMD (random) 95% CI	Weight %	WMD (random) 95% CI
Baik *et al*[12] 2008	18	6.90 (1.30)	18	8.70 (1.30)		41.56	-1.80 (-2.65, -0.95)
Patriti *et al*[11] 2009	29	11.90 (7.50)	37	9.60 (6.90)		21.11	2.30 (-1.22, 5.82)
Park *et al*[6] 2011	52	10.40 (4.70)	123	9.80 (3.80)		37.34	0.60 (-0.84, 2.04)
Total (95% CI)	99		178			100	-0.04 (-2.28, 2.20)

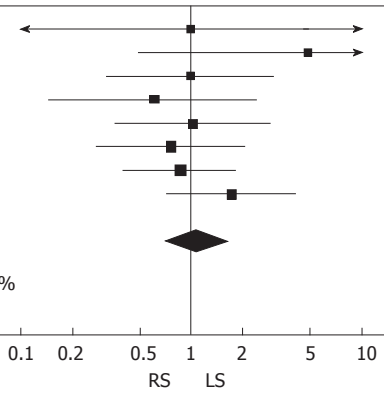
Test for heterogeneity: χ^2 = 11.49, df = 2 (P = 0.003), I^2 = 82.6%
Test for overall effect: Z = 0.03 (P = 0.97)

F

Review: Meta-analysis of robotic and laparoscopic surgery for treatment of rectal cancer
Comparison: 03 Intraoperative data
Outcome: 03 Complications

Study or sub-category	RS *n/N*	LS *n/N*	OR (fixed) 95% CI	Weight %	OR (fixed) 95% CI
Pigazzi *et al*[13] 2006	1/6	1/6		1.7	1.00 (0.05, 20.83)
Baik *et al*[12] 2008	4/18	1/18		1.58	4.86 (0.49, 48.57)
Patriti *et al*[11] 2009	7/29	9/37		12.21	0.99 (0.32, 3.08)
Bianchi *et al*[10] 2010	4/25	6/25		10.25	0.60 (0.15, 2.47)
Popescu *et al*[9] 2010	6/38	13/84		13.87	1.02 (0.36, 2.94)
Baek *et al*[7] 2011	9/41	11/41		17.47	0.77 (0.28, 2.11)
Kwak *et al*[8] 2011	18/59	20/59		28.27	0.86 (0.40, 1.85)
Park *et al*[6] 2011	10/52	15/123		14.65	1.71 (0.71, 4.12)
Total (95% CI)	268	393		100	1.05 (0.71, 1.55)

Total events: 59 (RS), 76 (LS)
Test for heterogeneity: χ^2 = 4.15, df = 7 (P = 0.76), I^2 = 0%
Test for overall effect: Z = 0.22 (P = 0.82)



G

Review: Meta-analysis of robotic and laparoscopic surgery for treatment of rectal cancer
Comparison: 03 Pathologic details
Outcome: 01 Lymph nodes harvested

Study or sub-category	*N*	RS mean (SD)	*N*	LS mean (SD)	WMD (fixed) 95% CI	Weight %	WMD (fixed) 95% CI
Baik *et al*[12] 2008	18	20.00 (9.10)	18	17.40 (10.60)		2.84	2.60 (-3.85, 9.05)
Patriti *et al*[11] 2009	29	10.30 (4.00)	37	11.20 (5.00)		25.05	-0.90 (-3.07, 1.27)
Popescu *et al*[9] 2010	38	11.37 (3.80)	84	11.07 (3.20)		61.25	0.30 (-1.09, 1.69)
Park *et al*[6] 2011	52	19.40 (10.20)	123	15.90 (10.10)		10.86	3.50 (0.20, 6.80)
Total (95% CI)	137		262			100	0.41 (-0.67, 1.50)

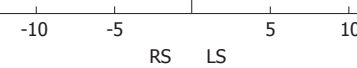
Test for heterogeneity: χ^2 = 5.24, df = 3 (P = 0.16), I^2 = 42.7%
Test for overall effect: Z = 0.74 (P = 0.46)

H

Review: Meta-analysis of robotic and laparoscopic surgery for treatment of rectal cancer
Comparison: 03 Pathologic details
Outcome: 02 Distal resection margin

Study or sub-category	*N*	RS mean (SD)	*N*	LS mean (SD)	WMD (random) 95% CI	Weight %	WMD (random) 95% CI
Baik *et al*[12] 2008	18	4.00 (1.10)	18	3.70 (1.10)		41.56	0.30 (-0.42, 1.02)
Patriti *et al*[11] 2009	29	2.10 (0.90)	37	4.50 (7.20)		12.24	-2.40 (-4.74, -0.06)
Park *et al*[6] 2011	52	2.80 (1.90)	123	3.20 (2.10)		45.14	-0.40 (-1.04, 0.24)
Total (95% CI)	99		178			100	-0.35 (-1.27, 0.58)

Test for heterogeneity: χ^2 = 5.62, df = 2 (P = 0.06), I^2 = 64.4%
Test for overall effect: Z = 0.73 (P = 0.46)



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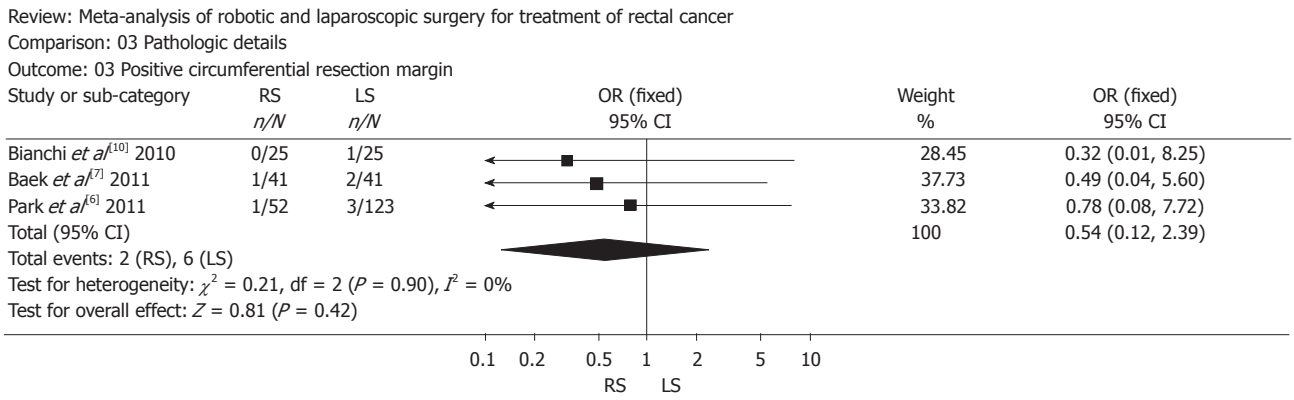

Figure 1 Forest plot displaying the results of the meta-analysis on operative time (A), blood loss (B), conversion (C), days to passing flatus (D), length of stay (E), complications (F), lymph nodes harvested (G), distal resection margin (H) and positive circumferential resection margin (I). RS: Robotic surgery; LS: Laparoscopic surgery; OR: Odds ratio; WMD: Weighted mean difference.

highly maneuverable EndoWrist instruments attached to the robotic arms.

Number of days to passing flatus was lower in the RS group than the LS group, meanwhile, length of stay was found to be no different between the two groups. However, analysis of the pooled data did not reveal any significant difference in this regard. These findings implied that the time required for patients to resume daily activities might not be shorter after RS than LS. There was no significant difference in complications when comparing RS and LS. On the contrary, it has been postulated that these characteristics of RS could make patients recover faster and reduce complications, because with the da Vinci surgical system, robotic arms are used for retraction and dissection during the total mesorectal excision procedure, and their use reduces unnecessary procedures and minimizes iatrogenic tissue injury during retraction. These findings are difficult to explain, and more advanced studies are needed before such conclusions can be drawn.

We postulated that specimen quality could be used as an indicator to predict long-term clinical oncological results. No significant differences were proved between RS and LS in the pathological details, including harvested lymph nodes, DRM and PCRM. The number of harvested lymph nodes, DRM, and PCRM did not differ significantly between the two groups in our meta-analysis. This demonstrated that RS could be performed safely and with a high success rate following oncological principles compared with LS. However, long-term follow-up evaluation is necessary to evaluate the exact oncological outcomes of RS for rectal cancer.

The cost of RS equipment is very high and likely to be a serious impediment to uptake in the foreseeable future[22]. However, it is important to perform a cost-effectiveness analysis between RS and LS. Only one trial has reported that the average total hospitalization costs were higher in the RS group ($83 915) than in the LS group ($62 601), and these differences were not statistically significant[7]. To the best of our knowledge, total hospitalization costs may be due to the greater expense and consumption of operating room resources such as space and the availability of skilled technical staff, and differ significantly between hospitals[23]. Therefore, insufficient data and great heterogeneity precluded a meta-analysis of cost-effectiveness.

Significant heterogeneity in those articles was observed in the operative time, blood loss, days to passing flatus, length of stay and DRM, which may be explained by the difference in skill, extension of lymph node dissection, and duration of learning curve. Regarding the heterogeneity between the articles, random-effect models were used in this meta-analysis.

The results of the present meta-analysis should be interpreted with caution because of several limitations. First, some data came from NRCTs, and the overall level of clinical evidence was low. It has been reported that NRCTs can either exaggerate or underestimate the magnitude of measured effects in a study of intervention regardless of quality scores[24]. However, Abrahama *et al*[25] have found that meta-analysis of well-designed NRCTs of surgical procedures was probably as accurate as that of RCTs. In fact, six studies included in the present study were NRCTs. Second, there was heterogeneity between the two groups, because it was impossible to match patient characteristics in all studies. We applied a random-effect model to take into consideration between-study variation, and it might have been expected to exert a limited influence. Finally, authors might be more likely to report positive results, and studies with significant outcomes were more likely to be published, so potential publication bias might have been present.

In conclusion, the results of this meta-analysis of 661 patients show that RS is superior to LS for rectal cancer in terms of conversion. Therefore, RS may be an alternative treatment for rectal cancer. Further studies are required to better define its role.

COMMENTS

Background

The da Vinci robotic system was introduced as the next advance in minimally

invasive surgery to overcome the technical limitations of laparoscopy, but robotic rectal surgery is controversial because of a lack of well-powered trials.

Research frontiers

Meta-analysis was used to evaluate the relative merits of robotic surgery (RS) and laparoscopic surgery (LS) for rectal cancer in this study.

Innovations and breakthroughs

The meta-analysis reported that RS had favorable outcomes considering conversion, compared with LS for rectal cancer. Meanwhile, operative time, blood loss, days to passing flatus, length of stay, complications and pathological details, including lymph nodes harvested, distal resection margin, and positive circumferential resection margin were similar between RS and LS. This is believed to be the first comprehensive meta-analysis comparing RS and LS for rectal cancer.

Applications

The results of this meta-analysis show that RS is superior to LS in terms of conversion. Therefore, RS may be an alternative treatment for rectal cancer.

Peer review

This paper addressed superiority of RS for rectal cancer, especially due to superior exposure and visualization of the intrapelvic field. This advantage means that surgeons complete the operation without conversion. This paper should be of interest to colorectal surgeons worldwide.

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BRIEF ARTICLE

Therapeutic effects of combined oxaliplatin and S-1 in older patients with advanced gastric cardiac adenocarcinoma

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Abstract

AIM: To evaluate the effects and safety of combination chemotherapy with oxaliplatin (L-OHP) and S-1 (SOX regimen) in older patients with advanced gastric cardiac adenocarcinoma (GCA).

METHODS: Seventy patients with advanced GCA were classified according to age into an older group (≥ 75 years) and a control group (< 75 years). The SOX regimen was administered to the two groups as follows: S-1 (40 mg/m^2 po bid) on days 1 to 14 followed by a 7-d off period, plus L-OHP (65 mg/m^2 iv) for 2 h on days 1 and 8 of a 21-d cycle. This regimen was repeated for four to six cycles. Response and swallow statuses were evaluated after two cycles (6 wk). Effects and toxicity were evaluated four weeks after chemotherapy was completed.

RESULTS: The response rate was 65.6% (21/32) in the older group and 68.4% (26/38) in the control group (χ^2 = 0.062 and P = 0.804). Improvement in swallowing was 78.1% (25/32) in the older group and 76.3% (29/38) in the control group (χ^2 = 0.032 and P = 0.857). Efficacy was 68.8% (22/32) in the older group and 65.8% (25/38) in the control group (χ^2 = 0.069 and P = 0.793). Toxicities were reversible and similar in both groups (P > 0.05).

CONCLUSION: The SOX regimen is an effective, safe and well-tolerated regimen for older patients with advanced GCA.

Key words: Gastric cardiac adenocarcinoma; Oxaliplatin; S-1; Treatment effect

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INTRODUCTION

With constant improvement in the quality of life in modern

society, people's life span has been prolonged. However, the incidence of elderly patients with gastric cardiac adenocarcinoma (GCA) is gradually increasing, and the majority of these patients have advanced disease when they are diagnosed. Thus, these patients have few opportunities for surgery[1]. The only available treatment choice for advanced GCA patients is systemic chemotherapy[2-4]. Although chemotherapy for advanced gastrointestinal cancer has been proven to be superior to best supportive care (BSC) in terms of survival and quality of life[5-7], there has been evidence supporting more serious adverse events observed among older patients than younger patients[8]. For these reasons, most older patients with metastasis are usually offered BSC and not chemotherapy[9]. However, patients who are 75 years old can still have a considerable number of years to survive (perhaps more than 10 years)[10]. Therefore, it is important to find a highly effective and minimally toxic chemotherapy regimen for elderly patients with advanced GCA.

In the last decade, 5-fluorouracil (5-FU) has been considered a cornerstone of therapy for advanced gastrointestinal cancer. Therefore, combining 5-FU with oxaliplatin (L-OHP) is logical because there is considerable evidence of preclinical synergy between the two agents[11]. S-1 is an orally active prodrug of 5-FU which is a fourth generation oral fluoropyrimidine[12]. Recent clinical studies have reported that S-1 in combination with L-OHP has a high response rate ranging from 53% to 59% and an excellent toxicity profile in the treatment of advanced gastric cancer[13-15]. In these studies, however, there were only a few patients of 75 years of age or older. Furthermore, few studies on the outcome of the S-1 and oxaliplatin (SOX) regimen in patients with GCA have been reported. Therefore, we designed this study to determine the response rate and toxicity profile of SOX regimen in GCA patients over the age of 75 years.

MATERIALS AND METHODS

Patients

GCA was confirmed in 70 patients by pathologic diagnosis in the First Affiliated Hospital, Henan University of Science and Technology from March 2008 to October 2010. All patients were treated with chemotherapy for the first time in this study, and they were experiencing symptoms such as difficulty in drinking, difficulty in eating, vomiting mucus, anemia, and emaciation. The degree of cardia stenosis was assessed using the Stooler Classification System[16] and the barium meal examination. The results of the barium meal examination are shown in Table 1. There were 54 cases of grade Ⅲ, 14 cases of grade Ⅳ, and 2 cases of grade Ⅴ dysphagia. All patients were classified as stage Ⅲ or Ⅳ according to the TNM staging, and they had Karnofsky Performance Status (KPS) scores greater than or equal to 60 points, predicted life spans greater than three months, no contraindications to chemotherapy, and no previous treatment with chemotherapy. Their routine blood examinations, electrocardiograms (ECGs), liver function, and kidney function were also normal. All patients were examined with a computed tomography (CT) before and after chemotherapy, and they were evaluated by the same physician.

Table 1 Degree of cardia stenosis

Clinical classifications	Diet conditions	Cardia diameters in the barium meal exam (mm)
Ⅰ	Ordinary diet	8-10
Ⅱ	Semi-liquid diet	6-8
Ⅲ	Liquid diet	4-6
Ⅳ	No drinking	2-4
Ⅴ	Saliva refluxing	0-2

According to the most recent World Health Organization (WHO) definition of aged people, people who are 65 to 74 years old are categorized as "young aged", and people who are 75 to 90 years old are classified as "older people". All the patients were divided into two groups as follows: patients older than 75 years were classified in the older group, and the remaining patients were classified in the control group. Of the 32 participants in the older group (ranging in age from 75 to 89 years old), 24 patients were male and 8 patients were female, with a median age of 79.5 years. Of the 38 participants in the control group (ranging in age from 55 to 74 years old), 29 patients were male and 9 patients were female with a median age of 64 years (Table 2).

Methods

The following chemotherapy program was used: L-OHP (65 mg/m^2 iv) was administered for 2 h on days 1 and 8; S-1 was orally administered at a dose of 40 mg/m^2 bid for 14 d (from the evening on day 1 until the morning on day 15); and a 7-d rest period followed the L-OHP and S-1 treatments in the 3-wk schedule. Treatment was repeated for four to six cycles. In every cycle, both omeprazole (40 mg iv bid) and tropisetron (5 mg iv qd) were administered before chemotherapy. Furthermore, large doses of oral vitamin B tablets were used to reduce side effects, and low doses of megestrol enhanced appetite and nutrition. Moreover, reconstituted cell colony-stimulating factor was given if needed. Participants were advised to avoid cold food, drinks and water. Blood, urine and stool routine examinations were carried out weekly, and ECG, liver function and kidney function were also checked weekly. Furthermore, a KPS score was determined weekly.

If patients had dysphagia to an extent greater than grade Ⅳ due to cardia stenosis, the stenosis was dilated with a conical Savary-Gilliard silica gel dilator one week before chemotherapy followed by insertion of a gastric canal. High protein and high vitamin liquid nasal feeds were then started. If the patient could swallow food after two chemotherapy cycles, the gastric canal was removed.

The sensitivity of the tumor to chemotherapy and improvement of dysphagia were evaluated after two cycles (6 wk). The effects and toxicity were evaluated at

Table 2 Patient characteristics at baseline, case (%)

Characteristics	Older group (n = 32)	Control group (n = 38)
Demography		
Male/female	24 (75)/8 (25)	29 (76.3)/9 (23.7)
Median age, yr (range)	79.5 (75-89)	64 (55-74)
Karnofsky performance status		
Median	80%	80%
100%	1 (3.1)	2 (5.3)
90%	10 (31.2)	13 (34.2)
80%	17 (53.2)	18 (47.4)
60%-70%	4 (12.5)	5 (13.1)
Weight loss > 5%	11 (34.4)	13 (34.2)
Cardia stenosis status		
Ⅰ-Ⅱ	0	0
Ⅲ	25 (78.1)	29 (76.3)
Ⅳ	6 (18.8)	8 (21.1)
Ⅴ	1 (3.1)	1 (2.6)
Histological grade		
G1-2	17 (53.1)	19 (50)
G3	12 (37.5)	14 (36.8)
Others (grade not stated)	3 (9.4)	5 (13.2)
Extent of disease		
Metastatic	10 (31.3)	12 (31.6)
Locally advanced	22 (68.7)	26 (68.4)
Metastatic site		
Lymph nodes	10 (31.3)	12 (31.6)
Liver	3 (9.4)	3 (7.9)
Peritoneum	1 (3.1)	2 (5.3)
Lung	0	1 (2.6)
Others	0	0
No. of metastatic sites		
1	6 (18.8)	6 (15.8)
⩾ 2	4 (12.5)	6 (15.8)

four weeks with a repeat CT and barium meal examination after the chemotherapy was completed.

Evaluation criteria

Evaluation criteria for chemotherapy sensitivity: The evaluation criteria for chemotherapy sensitivity we used were proposed in 1998 by the European Association of Cancer Research and Treatment, United States National Cancer Institute, and National Cancer Institute of Canada. These evaluation criteria are called the Response Evaluation Criteria In Solid Tumors[17]. Participants had a repeat CT scan with contrast two weeks after the completion of chemotherapy to evaluate the therapeutic effects of the chemotherapy according to the maximum diameters of each tumor. A complete response (CR) was defined as the complete disappearance of all lesions after treatment. A partial response (PR) was defined as a decrease greater than or equal to 30% in the maximum diameters of all tumors after treatment. Progressive disease (PD) was defined as an increase greater than 20% in the maximum diameters of tumors or the emergence of more than one new lesion after treatment. When the tumor diameters were between the diameters found in the PR and PD classifications (< 30% decrease or ⩽ 20% increase) after treatment, the effect was classified as stable disease.

Evaluation criteria for improvement of dysphagia: The evaluation criteria for symptom improvement were based on diet intake and the increase/decrease in cardia diameter. The symptoms were assessed using the barium meal examination[18] with the following classifications: CR, post-treatment cardia diameter two times greater than or equal to the pre-treatment cardia diameter with the patient capable of eating ordinary food; PR, post-treatment cardia diameter one time greater than the pre-treatment cardia diameter with the patient capable of eating semi-liquid food; no change (NC), an increase in the cardia diameter by less than 6 mm with the patient capable of eating only liquid food; and PD, a decrease in the cardia diameter with the patient unable to eat liquid food.

Evaluation criteria for short-term effects: Participants had CT scans in the first and fourth week after the chemotherapy session ended. The area of each tumor (referring to the product of the two longest vertical diameters) was measured before and after chemotherapy. The following evaluation criteria were used[19]: CR, complete disappearance of visible lesions for more than one month; PR, a decrease greater than 50% in the tumor for more than one month; NC, a decrease less than 50% or an increase less than 25% in the tumor for more than one month; and PD, one or more lesions increased by greater than 25% or the emergence of a new lump.

Evaluation criteria for side effects: Toxicities were divided into degrees from 0 to Ⅳ according to the WHO criteria for acute and subacute toxic reactions of antineoplastic agents[19].

Statistical analysis

SPSS 10.0 statistical software (SPSS Company, Chicago, Illinois, United States) was used to perform the χ^2 test to evaluate the data. P values less than 0.05 were considered statistically significant.

RESULTS

Chemotherapy sensitivity

A repeat CT of the epigastrium two weeks after starting chemotherapy with the SOX program measured changes in the diameter of the largest tumor and evaluated the sensitivity to chemotherapy of the older group and control group (Table 3).

Symptom (dysphagia) improvement

After two cycles of chemotherapy with the SOX program, an upper gastrointestinal barium meal examination was repeated. Changes in cardia diameters were measured and calculated, and patients were asked about their diets. Symptom improvement was evaluated and compared between groups (Table 4).

Short-term therapeutic effects

After one week and four weeks of chemotherapy with

Table 3 Comparisons of chemotherapy sensitivity, case (%)

Clinical groups	CR	PR	SD	PD	CR + PR
Older group (n = 32)	3 (9.4)	18 (56.2)	11 (34.4)	0	21 (65.6)[a]
Control group (n = 38)	4 (10.5)	22 (57.9)	12 (31.6)	0	26 (68.4)

[a]χ^2 = 0.062, P = 0.804 *vs* control group. CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

Table 4 Comparisons of symptom (dysphagia) improvement, case (%)

Clinical groups	CR	PR	NC	PD	CR + PR
Older group (n = 32)	5 (15.6)	20 (62.5)	7 (21.9)	0 (0)	25 (78.1)[a]
Control group (n = 38)	5 (13.2)	24 (63.2)	9 (23.7)	0 (0)	29 (76.3)

[a]χ^2 = 0.032, P = 0.857 *vs* control group. CR: Complete response; PR: Partial response; NC: No change; PD: Progressive disease.

Table 5 Comparisons of short-term chemotherapy effects, case (%)

Clinical groups	CR	PR	SD	PD	CR+PR
Older group (n = 32)	5 (15.6)	17 (53.2)	8 (25)	2 (6.2)	22 (68.8)[a]
Control group (n = 38)	5 (13.2)	20 (52.6)	10 (26.3)	3 (7.9)	25 (65.8)

[a]χ^2 = 0.069, P = 0.793 *vs* control group. CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

the SOX program, abdominal CTs were repeated. The maximum diameters of the tumors were measured, and the short-term therapeutic effects in both groups were evaluated (Table 5).

Side effects

The most frequent toxic therapy effects were hematological effects in both groups [grade 3 toxicity found in 13 patients (6 in the older group and 7 in the younger group)]. No grade 4 toxicity was reported. The L-OHP-related peripheral neuropathy appeared to be mild and reversible in the majority of cases. No severe cardiac toxicity or death was recorded among these patients during the study. Details of the side effects are shown in Table 6.

DISCUSSION

The health of the elderly varies from the health of younger patients. Older people are prone to having multiple organ dysfunctions, lower immunity, lower resistance to disease, and lower resistance to senile diseases, leading to reduced tolerance to chemotherapy and increased sensitivity to side effects of these drugs. Generally, caution is required when administering chemotherapy to older patients because they may not be able to tolerate a routine dose or may experience serious side effects. However, a suboptimal dose may not achieve the desired therapeutic effect. Therefore, many experts avoid treating elderly patients with chemotherapy[20].

Table 6 Comparisons of chemotherapy side effects, case (%)

Side effect	Older group (n = 32)			Control group (n = 38)			P[1] value
	I -IV	III	IV	I -IV	III	IV	
Leukopenia	25 (78.1)	3 (9.3)	0	28 (73.7)	4 (10.5)	0	0.666
Anemia	24 (75.0)	2 (6.2)	0	27 (71.1)	2 (5.3)	0	0.900
Thrombocytopenia	23 (71.9)	1 (3.1)	0	27 (71.1)	1 (2.6)	0	0.940
Fever	2 (6.3)	0	0	3 (7.9)	0	0	1.000
Oral mucositis	13 (40.6)	0	0	14 (36.8)	0	0	0.746
Nausea/vomiting	10 (31.3)	0	0	11 (28.9)	0	0	0.834
Diarrhea	14 (43.8)	0	0	16 (42.1)	0	0	0.890
Fatigue	21 (65.6)	2 (6.3)	0	23 (60.5)	1 (2.6)	0	0.660
Sensory neuropathy	18 (56.3)	0	0	21 (55.3)	0	0	0.934
Liver function (ALT/AST)	4 (12.5)	0	0	3 (7.9)	0	0	0.810
Renal function (BUN/Cr)	1 (3.1)	0	0	0	0	0	-
Hand-foot syndrome	0	0	0	0	0	0	-
Myocardial ischemia	0	0	0	0	0	0	-
Anaphylaxis	0	0	0	0	0	0	-

[1]P value for grade I -IV between older group and control group. ALT: Alanine transaminase; AST: Aspartate aminotransferase; BUN: Blood urea nitrogen; Cr: Creatine.

There is evidence[9,21], however, that older patients with advanced gastroesophageal carcinoma may benefit from chemotherapy. Tougeron *et al*[9] reported that palliative treatment is superior to BSC (6.7 ± 2.1 mo *vs* 1.8 ± 0.4 mo) in older patients (> 70 years of age) with advanced esophageal cancer. The effect of S-1 and cisplatin combination therapy in an 80-year-old patient with gastric carcinoma has been reported in a case study, and the histopathological examination of this patient revealed CR of the disease with no cancer cells[21]. Nevertheless, data regarding GCA is limited.

In this study, the SOX program was used to treat elderly people with advanced GCA to achieve the following goals: (1) to enhance the efficacy of treatment by using a new drug; (2) to reduce toxicity and improve tolerance; and (3) to create an opportunity for treatment in elderly patients with poor health.

S-1 is an effective derivative that combines tegafur with the following two modulators of 5-FU metabolism in a 1:0.4:1 molar ratio: 5-chloro-2,4-dihydroxypyridine (CDHP), a reversible inhibitor of dihydropyrimidine dehydrogenase (DPD), and potassium oxonate[12]. Tegafur, an oral prodrug of 5-FU, is gradually converted to 5-FU and is rapidly metabolized by DPD in the liver. The maximum concentration (Cmax) and area under the concentration-time curve (AUC) of 5-FU in plasma during S-1 treatment have been found to be higher than the steady state concentration and AUC of 5-FU in plasma during protracted intravenous infusion of 5-FU at a dose of 250 mg/m^2 per day[22]. Potassium oxonate is an orotate phosphoribosyl transferase inhibitor, which is primarily distributed to the gastrointestinal tract. This component of S-1 decreases incorporation of 5-fluorouridine triphosphate into RNA in the gastrointestinal mucosa, and it reduces the incidence of diarrhea. F-b-alanine (FBAL) is the main metabolite of 5-FU. FBAL and fluorocitrate are thought to cause the neurotoxic and

cardiotoxic effects of 5-FU by inhibiting the tricarboxylic acid cycle[22]. The CDHP component of S-1 inhibits DPD, which is the rate-limiting enzyme in the catabolic pathway of 5-FU. Consequently, the plasma FBAL concentration after oral administration of S-1 is significantly lower than the concentration after continuous infusion of 5-FU[12]. Therefore, the use of S-1 may decrease the incidence of neurotoxicity and cardiotoxicity. Ajani *et al*[23] reported significant safety advantages in the S-1/cisplatin treatment as compared with the infusional fluorouracil/cisplatin treatment for advanced gastric or gastroesophageal adenocarcinoma. They reported the following frequencies resulting from the two treatments: grade 3/4 neutropenia (32.3% and 63.6%, respectively), stomatitis (1.3% and 13.6%, respectively), and hypokalemia (3.6% and 10.8%, respectively).

L-OHP[24-25] is a third generation platinum anticancer drug developed to improve tolerability and ease of administration when compared to cisplatin. The rate at which L-OHP combines with DNA in the body is more than 10 times faster than cisplatin. L-OHP adheres more strongly to DNA, and it has a stronger cytotoxic effect than cisplatin and carboplatin. In addition, the unique diaminocyclohexane group in oxaliplatin avoids some of the resistance mechanisms developed against cisplatin, such as the mismatch repair defect and bypass replication mechanism. A phase Ⅲ trial[26] for metastatic gastroesophageal adenocarcinoma has been conducted, with a treatment of fluorouracil and leucovorin combined with either oxaliplatin [fluorouracil, leucovorin and oxaliplatin (FLO)] or cisplatin [fluorouracil, leucovorin and cisplatin (FLP)] every two weeks. The results of this trial demonstrated that serious adverse events associated with FLO are significantly less than the events associated with FLP (9% and 19%, respectively) and that the median progression-free survival (PFS) improves with FLO when compared to FLP (5.8 mo and 3.9 mo, respectively). This trial also demonstrated that treatment with FLO results in significantly superior response rates (41.3% and 16.7%, respectively), improved median PFS (6.0 mo and 3.1 mo, respectively) and improved overall survival (13.9 mo and 7.2 mo, respectively) when compared to treatment with FLP in patients older than 65 years.

Studies have shown that L-OHP and S-1 are highly active against cancer and that they have a favorable toxicity profile. Furthermore, studies have also shown that L-OHP and S-1 are expected to replace cisplatin and fluorouracil, respectively, as a first-line treatment for advanced gastric cancer[13-15]. Moreover, the SOX program may be considered for treatment of older people because of the greater efficacy and low toxicity of this regimen when compared to cisplatin and fluorouracil.

The SOX regimen in this study resulted in no significant differences between the older and control groups with regard to chemotherapy sensitivity (65.6% and 68.4%, respectively, $P = 0.804$), symptom improvement (78.1% and 76.3%, respectively, $P = 0.857$), and short-term therapeutic effects (68.8% and 65.8%, respectively, $P = 0.793$). More severe side effects caused by the SOX regimen were not detected among the elderly patients when compared to the younger patients, and these side effects did not have a significant effect on treatment administration or quality of life. Therefore, these results suggest that there are treatment options available for elderly patients with cardia obstruction who cannot eat and that there is still an opportunity for these patients to survive if they can get adequate nutrition through nasal feeds.

In summary, the SOX regimen for advanced GCA has high efficacy and mild toxicity, and it can increase the survival and life span of patients with GCA. Moreover, the SOX regimen is a safe chemotherapy program for elderly patients in poor health. Therefore, it is not necessary to entirely avoid chemotherapy in elderly patients with advanced GCA because of their age. Instead, treatment recommendations should consider physiological age and standard KPS score. It is also reasonable to initiate chemotherapy if the patient can obtain sufficient nutrition (e.g., through nasal feeding). However, chemotherapy should not be administered to patients with KPS scores less than 60 points.

In this study, the therapeutic effects of the SOX regimen in both groups were higher than those reported in previous studies of patients with gastric cancer[13-15], which may have been due to the fact that this combination therapy was the first time any of the patients in this study were treated with chemotherapy, resulting in a higher sensitivity and minimal resistance to treatment. Other studies have included patients who had relapsed or failed treatment. Moreover, most of the patients in this study were classified as having stage Ⅲb GCA with only locally advanced cancer. In this study, there were only a few extensive cases of metastasized cancer. Additionally, the SOX program may be more effective at treating GCA than other types of gastric cancer.

COMMENTS

Background

The morbidity of gastric cardiac adenocarcinoma (GCA) in elderly people is gradually increasing, and most elderly GCA patients suffer from advanced carcinoma. Therefore, the opportunity for surgery is low, and only systemic chemotherapy is available for these patients. However, many experts disagree with treating elderly patients with chemotherapy because more serious adverse events have been observed in older patients than in younger patients.

Research frontiers

In the last decade, 5-fluorouracil (5-FU) has been considered a cornerstone for treating advanced gastrointestinal cancers. S-1 is a new orally active prodrug of 5-FU, and clinical studies with S-1/L-OHP (SOX regimen) have reported a high response rate ranging from 53% to 59% and an excellent toxicity profile in the treatment of advanced gastric cancer. In these clinical studies, however, there were only a few patients who were 75 years of age or older. Moreover, only a few studies on the outcome of the SOX regimen in patients with GCA have been reported.

Innovations and breakthroughs

This is the first study to evaluate the effects and safety of the SOX regimen in older patients with GCA. The study showed that the SOX regimen is a safe chemotherapy program for elderly patients with advanced GCA and that this regimen provides a treatment option for elderly patients with GCA.

Applications

The SOX regimen may be an ideal strategy in the future for treatment of older patients with advanced GCA.

Peer review

It is a very interesting topic for the readers.

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CASE REPORT

Enterovenous fistulization: A rare complication of Crohn's disease

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Abstract

The presence of hepatic portal venous gas (HPVG) is associated with numerous diseases, and has been regarded as a serious, even catastrophic condition. However, anecdotal reports mention that some patients with inflammatory bowel disease (IBD), who developed HPVG after diagnostic examinations of the colon, were successfully managed with antibiotic therapy and have followed benign courses. In contrast, among IBD patients, the development of HPVG is rarely caused by enterovenous fistula. We describe a 32-year-old man with Crohn's ileocolitis who presented with hypotension and fever associated with HPVG, as well as superior mesenteric vein thrombosis, possibly caused by enterovenous fistula, who was successfully managed by surgery. We also review the literature concerning portal venous gas associated with Crohn's disease.

Key words: Crohn's disease; Enterovenous fistula; Portal venous gas

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INTRODUCTION

Hepatic portal venous gas (HPVG), as first described in 1955[1], has been reported in many illnesses, ranging from benign conditions to potentially lethal diseases that require urgent surgical intervention[2]. Overall mortality depends on the underlying disease[3].

Among the various diseases, HPVG associated with Crohn's disease (CD) has rarely been described. With regard to the treatment of patients with CD complicated with HPVG, some anecdotal reports suggested that conservative treatment with antibiotics might be effective. However, early recognition and urgent operative intervention at the time of presentation are still important in patients with complicated CD. We report a 32-year-old patient with CD who presented with fever and hypotension associated with HPVG and mesenteric vein thrombosis who was successfully managed surgically. To our knowledge, this is the first report of HPVG and mesenteric vein thrombosis possibly caused by enterovenous fistula complicated by CD in South Korea. The literature concerning PVG associated with CD is also reviewed.

CASE REPORT

A 32-year-old man was referred for evaluation of mesenteric and portal venous gas detected by abdominal

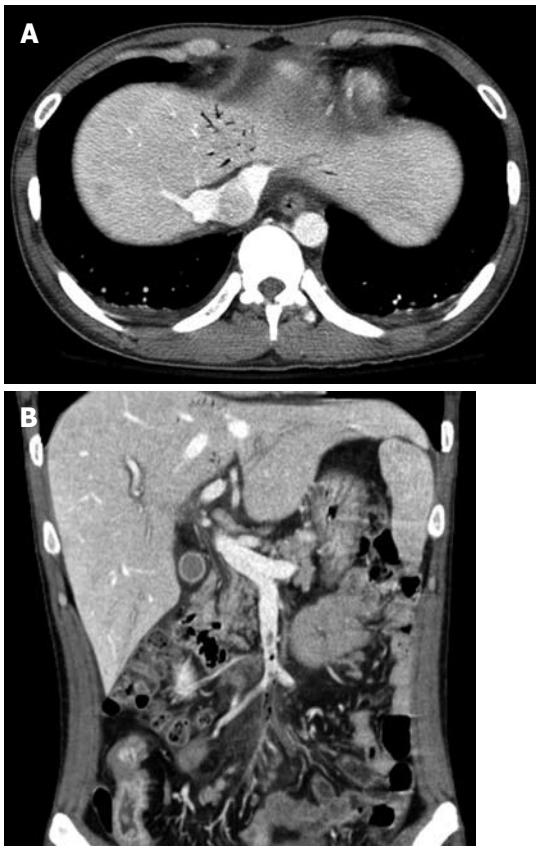

Figure 1 Computed tomography of the abdomen showing evidence of (A) portal venous gas, and (B) superior mesenteric venous gas and thrombus.

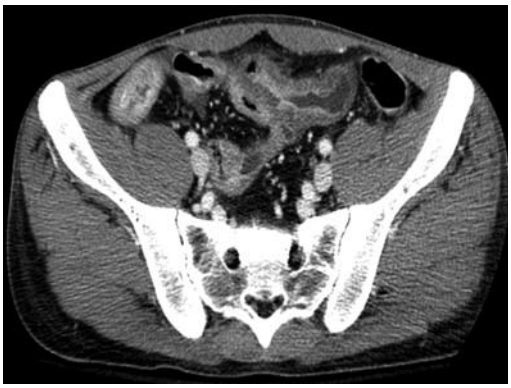

Figure 2 Computed tomography of the abdomen showing evidence of prominent wall thickening with perienteric infiltration in the mid-ileum.

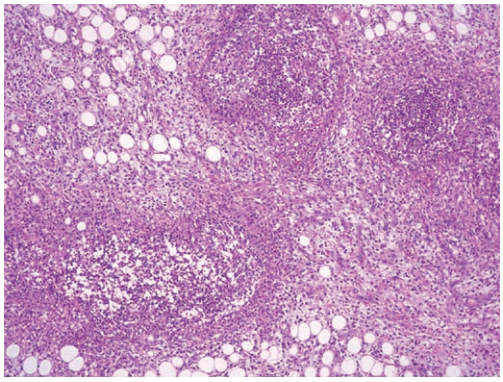

Figure 3 Photography of the pathology, showing the accumulation of many neutrophils in the intravascular lumen (hematoxylin and eosion stain, × 100).

computerized tomography (CT). The patient had complained of lower abdominal pain, nausea, vomiting, and fever. He was diagnosed with CD complicated by perianal fistula 12 years prior to admission, and was treated with azathioprine (25 mg/d) and 5-aminosalicylate (ASA; 3.2 g/d). He had been in his usual state of health on azathioprine and ASA until abdominal pain developed 21 d before admission at which time budesonide (9 mg/d) was added to his regimen of azathioprine and ASA. He continued on all three medications for three weeks until developing fever 2 d prior to admission. At that time, an abdominal CT showed mesenteric and portal venous gas. He was then referred to this hospital.

At admission, his body temperature was 38.5 ℃, blood pressure was 82/47 mmHg, and pulse rate was 105 beats per minute. There was moderate tenderness on the lower abdomen, but no abdominal distension or signs of peritoneal irritation. His white blood cell count was 4600/mm^3 (normal range 4000-10 000/mm^3) with 87% segmented forms. Protein was 4.9 g/dL (normal range 3.5-5.2 g/dL), albumin was 2.1 g/dL (normal range 6.0-8.5 g/dL), C-reactive protein was 21.19 mg/dL (normal range < 0.3 mg/dL), prothrombin time was 1.41 international normalized ratio (INR) (normal range 0.84-1.21 INR), and activated partial thromboplastin time was 37.3 s (normal range 26.3-39.4 s). Plain abdominal films were unremarkable. Abdominopelvic CT revealed the presence of air bubbles in the hepatic portal vein, superior mesenteric vein (SMV), and its contributaries. Intravenous thrombi were also noted (Figure 1). With regard to the bowel, multiple segments of the small bowel were thickened in the mid to the distal ileum and distal colon, especially in a 20 cm segment of the mid-ileum, near the mesenteric venous gas (Figure 2). The abdominopelvic CT findings were compatible with CD complicated with thrombophlebitis of the SMV. Hence, an explorative laparotomy was performed. At operation, a small amount of serous ascites was noted throughout the peritoneal cavity with multiple strictures and areas of inflammation which were also noted along the mid to distal ileum. Moreover, a short segment of mid-ileum showed phlegmonous change. Ileocecal resection was performed. Pathologic examination disclosed transmural inflammation with lymphoid aggregates, multiple microgranulomas and a fistulous tract. In addition, many neutrophils had accumulated in the intravascular space, suggesting the presence of an enterovenous fistula (Figure 3).

After surgery, the patient had an uneventful recovery and was discharged 9 d post surgery. The patient has been on 3 g of mesalazine and 25 mg of 6-mercaptopurine for his colonic disease and has been well for 4 mo after surgery.

DISCUSSION

HPVG has been reported in association with numerous conditions in adults, including intestinal ischemia or necrosis[4], intra-abdominal abscess[5], diverticulitis[6], pneumatosis intestinalis[7], and blunt trauma[8]. When HPVG occurred in association with necrotic bowel, the overall mortality rate rose to about 75%[9]. Thus, in the past, HPVG has been considered as an indicator for urgent surgical intervention with a poor prognosis. However, the development of highly advanced imaging techniques enables earlier detection of potentially severe pathologies, such as bowel ischemia, and allows prompt diagnosis and treatment, which results in significantly reduced mortality rates[10]. HPVG is not always a surgical condition, and its treatment should be based on the underlying disease and the patient's current clinical condition. HPVG in patients with inflammatory bowel disease can be caused by mucosal damage alone, or can occur in combination with bowel distension, sepsis, invasion by gas-producing bacteria, or after colonoscopy, upper gastrointestinal barium examination, barium enema or blunt abdominal trauma. Not all of these conditions require surgical intervention, especially in the absence of peritoneal signs or free gas in the peritoneal space[2].

The prognosis is related to the pathology itself and is not influenced by the presence of HPVG. Among 182 case studies reported by Kinoshita *et al*[2], patients with ulcerative colitis or CD comprised 4% of the total. To our knowledge, 21 cases of PVG associated with CD were reported in the English literature[11-13].

The formation of HPVG in patients with CD can be explained by the following hypothesis; first, elevated intracolonic pressure can permit bowel gas or gas-forming bacteria to gain access to the portal venous circulation. Elevated intracolonic pressure caused by blunt abdominal trauma, or by diagnostic procedures such as colonoscopy or barium enema occurred in 2 and 6 out of 21 patients, respectively, that had relatively benign clinical courses and rarely needed surgical intervention; only one of the eight required surgery. Second, the enterovenous fistula, which is an extremely rare complication of CD, can directly transfer bowel gas to the portal venous system. To date, HPVG associated with enterovenous fistula has been reported in 2 patients[14,15]. Two CD patients with enterovenous fistula required surgery, and one died due to sepsis. Third, HPVG can be the result of mucosal injury and sepsis associated with bowel inflammation and portal pyemia. Mucosal damage secondary to bowel inflammation provides an entry for intraluminal gas into the portal venous system. In addition, sepsis alone without necrotic bowel can be the cause of HPVG[9]. The remaining 11 patients included in that study, who had no identifiable predisposing factors, may have developed HPVG by this mechanism.

In our patient, the second mechanism is the most plausible explanation for HPVG based on surgical pathology and CT findings. Although there was no definite communication between bowel and vessel shown on the abdominal CT scan, a thrombosed vessel along with a thickened bowel suggests possible communication. The fistula between the small bowel and small branches of the superior mesenteric vein was not easily identified on CT because of its small size. Furthermore, histopathology showed many neutrophils accumulated in the intravascular space, an occluded small mesenteric vein caused by inflammatory thrombus, and no evidence of bowel ischemia. Based on the clinical features, histopathology, and imaging findings, mesenteric venous thrombosis was not a plausible diagnosis.

In eleven patients without a predisposing condition, eight (73%) presented with signs of intra-abdominal catastrophe or systemic toxicity and required surgery. One of eight died of disseminated cytomegalovirus infection, and the remaining three out these eleven patients were conservatively managed.

Two cases were reported with a combination of PVG and thrombophlebitis of the portal vein or SMV. These patients presented with septic shock and needed surgical treatment[11,16]. Although PVG itself is not a prognostic indicator, PVG combined with thrombophlebitis of the portal or mesenteric veins can be regarded as an indicator of poor prognosis.

Although the incidence of CD in Asians is still much lower than in Western patients, recent studies have reported that the incidence of CD in Asian populations is gradually increasing[17,18]. As the number of patients with CD increases, the proportion of complicated CD patients also increases. Although PVG accompanied by mesenteric venous thrombosis occurs very rarely in patients with CD, it complicates treatment decisions for the gastroenterologist. Thus, we should exercise caution regarding the clinical management in cases of CD with HPVG. HPVG associated with CD does not always mandate surgical intervention, especially in the absence of peritoneal signs or free intraperitoneal gas. Those patients found to have significant intestinal pathology, as in this case, as seen from imaging studies, or all other symptomatic patients who do not improve after medical treatment, should undergo urgent laparotomy[10]. The clinical course seems to be associated with predisposing factors, such as in this case, where hypotension and fever necessitated urgent surgery.

In conclusion, the finding of PVG is not always an indication for surgical intervention in CD, but PVG caused by enterovenous fistula requires urgent surgical treatment.

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World Journal of Gastroenterology

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Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011 Miami, FL 33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium 2011, San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

January 28-29, 2011
9. Gastro Forum München, Munich, Germany

February 4-5, 2011
13th Duesseldorf International Endoscopy Symposium, Duesseldorf, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference, Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week 2011, Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation, Dublin, Ireland

February 24-26, 2011
2nd International Congress on Abdominal Obesity, Buenos Aires, Brazil

February 24-26, 2011
International Colorectal Disease Symposium 2011, Hong Kong, China

February 26-March 1, 2011
Canadian Digestive Diseases Week, Westin Bayshore, Vancouver, British Columbia, Canada

February 28-March 1, 2011
Childhood & Adolescent Obesity: A whole-system strategic approach, Abu Dhabi, United Arab Emirates

March 3-5, 2011
42nd Annual Topics in Internal Medicine, Gainesville, FL 32614, United States

March 7-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings, Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011, Birmingham, England, United Kingdom

March 17-19, 2011
41. Kongress der Deutschen Gesellschaft für Endoskopie und Bildgebende Verfahren e.V., Munich, Germany

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011, Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform, Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011 Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease, San Diego, CA 94143, United States

April 6-7, 2011
IBS-A Global Perspective, Pfister Hotel, 424 East Wisconsin Avenue, Milwaukee, WI 53202, United States

April 7-9, 2011
International and Interdisciplinary Conference Excellence in Female Surgery, Florence, Italy

April 15-16, 2011
Falk Symposium 177, Endoscopy Live Berlin 2011 Intestinal Disease Meeting, Stauffenbergstr. 26, 10785 Berlin, Germany

April 18-22, 2011
Pediatric Emergency Medicine: Detection, Diagnosis and Developing Treatment Plans, Sarasota, FL 34234, United States

April 20-23, 2011
9th International Gastric Cancer Congress, COEX, World Trade Center, Samseong-dong, Gangnam-gu, Seoul 135-731, South Korea

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011
Neurology Updates for Primary Care, Sarasota, FL 34230-6947, United States

April 28-30, 2011
4th Central European Congress of Surgery, Budapest, Hungary

May 7-10, 2011
Digestive Disease Week, Chicago, IL 60446, United States

May 12-13, 2011
2nd National Conference Clinical Advances in Cystic Fibrosis, London, England, United Kingdom

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671 Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course, Venise, Italy

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn, Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011, Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011
International Scientific Conference on Probiotics and Prebiotics-IPC2011, Kosice, Slovakia

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer, Barcelona, Spain

June 29-2, 2011
XI Congreso Interamericano de Pediatria "Monterrey 2011", Monterrey, Mexico

September 2-3, 2011 Falk Symposium 178, Diverticular Disease, A Fresh Approach to a Neglected Disease, Gürzenich Cologne, Martinstr. 29-37, 50667 Cologne, Germany

September 10-11, 2011
New Advances in Inflammatory Bowel Disease, La Jolla, CA 92093, United States

September 10-14, 2011
ICE 2011-International Congress of Endoscopy, Los Angeles Convention Center, 1201 South Figueroa Street Los Angeles, CA 90015, United States

September 30-October 1, 2011
Falk Symposium 179, Revisiting IBD Management: Dogmas to be Challenged, Sheraton Brussels Hotel, Place Rogier 3, 1210 Brussels, Belgium

October 19-29, 2011
Cardiology & Gastroenterology | Tahiti 10 night CME Cruise, Papeete, French Polynesia

October 22-26, 2011
19th United European Gastroenterology Week, Stockholm, Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting & Postgraduate Course, Washington, DC 20001, United States

November 11-12, 2011
Falk Symposium 180, IBD 2011: Progress and Future for Lifelong Management, ANA Interconti Hotel, 1-12-33 Akasaka, Minato-ku, Tokyo 107-0052, Japan

December 1-4, 2011
2011 Advances in Inflammatory Bowel Diseases/Crohn's & Colitis Foundation's Clinical & Research Conference, Hollywood, FL 34234, United States

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GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a weekly, open-access (OA), peer-reviewed journal supported by an editorial board of 1144 experts in gastroenterology and hepatology from 60 countries.

The biggest advantage of the OA model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the value to the readers, authors and society.

Maximization of personal benefits

The role of academic journals is to exhibit the scientific levels of a country, a university, a center, a department, and even a scientist, and build an important bridge for communication between scientists and the public. As we all know, the significance of the publication of scientific articles lies not only in disseminating and communicating innovative scientific achievements and academic views, as well as promoting the application of scientific achievements, but also in formally recognizing the "priority" and "copyright" of innovative achievements published, as well as evaluating research performance and academic levels. So, to realize these desired attributes of *WJG* and create a well-recognized journal, the following four types of personal benefits should be maximized. The maximization of personal benefits refers to the pursuit of the maximum personal benefits in a well-considered optimal manner without violation of the laws, ethical rules and the benefits of others. (1) Maximization of the benefits of editorial board members: The primary task of editorial board members is to give a peer review of an unpublished scientific article via online office system to evaluate its innovativeness, scientific and practical values and determine whether it should be published or not. During peer review, editorial board members can also obtain cutting-edge information in that field at first hand. As leaders in their field, they have priority to be invited to write articles and publish commentary articles. We will put peer reviewers' names and affiliations along with the article they reviewed in the journal to acknowledge their contribution; (2) Maximization of the benefits of authors: Since *WJG* is an open-access journal, readers around the world can immediately download and read, free of charge, high-quality, peer-reviewed articles from *WJG* official website, thereby realizing the goals and significance of the communication between authors and peers as well as public reading; (3) Maximization of the benefits of readers: Readers can read or use, free of charge, high-quality peer-reviewed articles without any limits, and cite the arguments, viewpoints, concepts, theories, methods, results, conclusion or facts and data of pertinent literature so as to validate the innovativeness, scientific and practical values of their own research achievements, thus ensuring that their articles have novel arguments or viewpoints, solid evidence and correct conclusion; and (4) Maximization of the benefits of employees: It is an iron law that a first-class journal is unable to exist without first-class editors, and only first-class editors can create a first-class academic journal. We insist on strengthening our team cultivation and construction so that every employee, in an open, fair and transparent environment, could contribute their wisdom to edit and publish high-quality articles, thereby realizing the maximization of the personal benefits of editorial board members, authors and readers, and yielding the greatest social and economic benefits.

Aims and scope

The major task of *WJG* is to report rapidly the most recent results in basic and clinical research on esophageal, gastrointestinal, liver, pancreas and biliary tract diseases, *Helicobacter pylori*, endoscopy and gastrointestinal surgery, including: gastroesophageal reflux disease, gastrointestinal bleeding, infection and tumors; gastric and duodenal disorders; intestinal inflammation, microflora and immunity; celiac disease, dyspepsia and nutrition; viral hepatitis, portal hypertension, liver fibrosis, liver cirrhosis, liver transplantation, and metabolic liver disease; molecular and cell biology; geriatric and pediatric gastroenterology; diagnosis and screening, imaging and advanced technology.

Columns

The columns in the issues of *WJG* will include: (1) Editorial: To introduce and comment on major advances and developments in the field; (2) Frontier: To review representative achievements, comment on the state of current research, and propose directions for future research; (3) Topic Highlight: This column consists of three formats, including (A) 10 invited review articles on a hot topic, (B) a commentary on common issues of this hot topic, and (C) a commentary on the 10 individual articles; (4) Observation: To update the development of old and new questions, highlight unsolved problems, and provide strategies on how to solve the questions; (5) Guidelines for Basic Research: To provide guidelines for basic research; (6) Guidelines for Clinical Practice: To provide guidelines for clinical diagnosis and treatment; (7) Review: To review systemically progress and unresolved problems in the field, comment on the state of current research, and make suggestions for future work; (8) Original Article: To report innovative and original findings in gastroenterology; (9) Brief Article: To briefly report the novel and innovative findings in gastroenterology and hepatology; (10) Case Report: To report a rare or typical case; (11) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (12) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (13) Guidelines: To introduce consensuses and guidelines reached by international and national academic authorities worldwide on basic research and clinical practice gastroenterology and hepatology.

Name of journal

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SPECIAL STATEMENT

All articles published in this journal represent the viewpoints of the authors except where indicated otherwise.

Biostatistical editing
Statistical review is performed after peer review. We invite an expert in Biomedical Statistics from to evaluate the statistical method used in the paper, including *t*-test (group or paired comparisons), chi-squared test, Ridit, probit, logit, regression (linear, curvilinear, or stepwise), correlation, analysis of variance, analysis of covariance, *etc*. The reviewing points include: (1) Statistical methods should be described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only homogeneous data can be averaged. Standard deviations are preferred to standard errors. Give the number of observations and subjects (*n*). Losses in observations, such as drop-outs from the study should be reported; (4) Values such as ED50, LD50, IC50 should have their 95% confidence limits calculated and compared by weighted probit analysis (Bliss and Finney); and (5) The word 'significantly' should be replaced by its synonyms (if it indicates extent) or the *P* value (if it indicates statistical significance).

Conflict-of-interest statement
In the interests of transparency and to help reviewers assess any potential bias, *WJG* requires authors of all papers to declare any competing commercial, personal, political, intellectual, or religious interests in relation to the submitted work. Referees are also asked to indicate any potential conflict they might have reviewing a particular paper. Before submitting, authors are suggested to read "Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Ethical Considerations in the Conduct and Reporting of Research: Conflicts of Interest" from International Committee of Medical Journal Editors (ICMJE), which is available at: http://www.icmje.org/ethical_4conflicts.html.

Sample wording: [Name of individual] has received fees for serving as a speaker, a consultant and an advisory board member for [names of organizations], and has received research funding from [names of organization]. [Name of individual] is an employee of [name of organization]. [Name of individual] owns stocks and shares in [name of organization]. [Name of individual] owns patent [patent identification and brief description].

Statement of informed consent
Manuscripts should contain a statement to the effect that all human studies have been reviewed by the appropriate ethics committee or it should be stated clearly in the text that all persons gave their informed consent prior to their inclusion in the study. Details that might disclose the identity of the subjects under study should be omitted. Authors should also draw attention to the Code of Ethics of the World Medical Association (Declaration of Helsinki, 1964, as revised in 2004).

Statement of human and animal rights
When reporting the results from experiments, authors should follow the highest standards and the trial should conform to Good Clinical Practice (for example, US Food and Drug Administration Good Clinical Practice in FDA-Regulated Clinical Trials; UK Medicines Research Council Guidelines for Good Clinical Practice in Clinical Trials) and/or the World Medical Association Declaration of Helsinki. Generally, we suggest authors follow the lead investigator's national standard. If doubt exists whether the research was conducted in accordance with the above standards, the authors must explain the rationale for their approach and demonstrate that the institutional review body explicitly approved the doubtful aspects of the study.

Before submitting, authors should make their study approved by the relevant research ethics committee or institutional review board. If human participants were involved, manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and appropriate informed consent of each. Any personal item or information will not be published without explicit consents from the involved patients. If experimental animals were used, the materials and methods (experimental procedures) section must clearly indicate that appropriate measures were taken to minimize pain or discomfort, and details of animal care should be provided.

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Manuscripts should be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Number all pages consecutively, and start each of the following sections on a new page: Title Page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables, Figures, and Figure Legends. Neither the editors nor the publisher are responsible for the opinions expressed by contributors. Manuscripts formally accepted for publication become the permanent property of Baishideng Publishing Group Co., Limited, and may not be reproduced by any means, in whole or in part, without the written permission of both the authors and the publisher. We reserve the right to copy-edit and put onto our website accepted manuscripts. Authors should follow the relevant guidelines for the care and use of laboratory animals of their institution or national animal welfare committee. For the sake of transparency in regard to the performance and reporting of clinical trials, we endorse the policy of the ICMJE to refuse to publish papers on clinical trial results if the trial was not recorded in a publicly-accessible registry at its outset. The only register now available, to our knowledge, is http://www.clinicaltrials.gov sponsored by the United States National Library of Medicine and we encourage all potential contributors to register with it. However, in the case that other registers become available you will be duly notified. A letter of recommendation from each author's organization should be provided with the contributed article to ensure the privacy and secrecy of research is protected.

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MANUSCRIPT PREPARATION

All contributions should be written in English. All articles must be submitted using word-processing software. All submissions must be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Style should conform to our house format. Required information for each of the manuscript sections is as follows:

Title page

Title: Title should be less than 12 words.

Running title: A short running title of less than 6 words should be provided.

Authorship: Authorship credit should be in accordance with the standard proposed by ICMJE, based on (1) substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; and (3) final approval of the version to be published. Authors should meet conditions 1, 2, and 3.

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Author contributions: The format of this section should be: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed the research; Wang CL, Zou CC, Hong F and Wu XM performed the research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed the data; and Wang CL, Liang L and Fu JF wrote the paper.

Supportive foundations: The complete name and number of supportive foundations should be provided, e.g. Supported by National Natural Science Foundation of China, No. 30224801

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Peer reviewers: All articles received are subject to peer review. Normally, three experts are invited for each article. Decision for acceptance is made only when at least two experts recommend an article for publication. Reviewers for accepted manuscripts are acknowledged in each manuscript, and reviewers of articles which were not accepted will be acknowledged at the end of each issue. To ensure the quality of the articles published in *WJG*, reviewers of accepted manuscripts will be announced by publishing the name, title/position and institution of the reviewer in the footnote accompanying the printed article. For example, reviewers: Professor Jing-Yuan Fang, Shanghai Institute of Digestive Disease, Shanghai, Affiliated Renji Hospital, Medical Faculty, Shanghai Jiaotong University, Shanghai, China; Professor Xin-Wei Han, Department of Radiology, The First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan Province, China; and Professor Anren Kuang, Department of Nuclear Medicine, Huaxi Hospital, Sichuan University, Chengdu, Sichuan Province, China.

Abstract

There are unstructured abstracts (no more than 256 words) and structured abstracts (no more than 480). The specific requirements for structured abstracts are as follows:

An informative, structured abstracts of no more than 480 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections. AIM (no more than 20 words): Only the purpose should be included. Please write the aim as the form of "To investigate/study/…"; MATERIALS AND METHODS (no more than 140 words); RESULTS (no more than 294 words): You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, e.g. 6.92 ± 3.86 *vs* 3.61 ± 1.67, $P < 0.001$; CONCLUSION (no more than 26 words).

Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Text

For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm.

Illustrations

Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: http://www.wjgnet.com/1007-9327/13/4520.pdf; http://www.wjgnet.com/1007-9327/13/4554.pdf; http://www.wjgnet.com/1007-9327/13/4891.pdf; http://www.wjgnet.com/1007-9327/13/4986.pdf; http://www.wjgnet.com/1007-9327/13/4498.pdf. Keeping all elements compiled is necessary in line-art image. Scale bars should be

used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A:...; B:...; C:...; D:...; E:...; F:...; G: …*etc.* It is our principle to publish high resolution-figures for the printed and E-versions.

Tables

Three-line tables should be numbered 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement, but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

Notes in tables and illustrations

Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of P values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of P values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

REFERENCES

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The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability[1,2]". If references are cited directly in the text, they should be put together within the text, for example, "From references[19,22-24], we know that...".

When the authors write the references, please ensure that the order in text is the same as in the references section, and also ensure the spelling accuracy of the first author's name. Do not list the same citation twice.

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Pleased provide PubMed citation numbers to the reference list, e.g. PMID and DOI, which can be found at http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed and http://www.crossref.org/SimpleTextQuery/, respectively. The numbers will be used in E-version of this journal.

Style for journal references

Authors: the name of the first author should be typed in boldfaced letters. The family name of all authors should be typed with the initial letter capitalized, followed by their abbreviated first and middle initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). The title of the cited article and italicized journal title (journal title should be in its abbreviated form as shown in PubMed), publication date, volume number (in black), start page, and end page [PMID: 11819634 DOI: 10.3748/wjg.13.5396].

Style for book references

Authors: the name of the first author should be typed in boldfaced letters. The surname of all authors should be typed with the initial letter capitalized, followed by their abbreviated middle and first initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR) Book title. Publication number. Publication place: Publication press, Year: start page and end page.

Format

Journals

English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

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Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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