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ABOUT COVER

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AIM AND SCOPE

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EDITORIAL

Biosimilars: Review of current applications, obstacles, and their future in medicine

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Abstract

Biosimilars are a growing drug class designed to be used interchangeably with biologics. Biologics are created in living cells and are typically large, complex proteins that may have a variety of uses. Within the field of gastroenterology alone, biologics are used to treat inflammatory bowel diseases, cancers, and endocrine disorders. While biologics have proven to be effective in treating or managing many diseases, patient access is often limited by high costs. The development of biosimilars is an attempt to reduce treatment costs. Biosimilars must be nearly identical to their reference biologics in terms of efficacy, side effect risk profile, and immunogenicity. Although the manufacturing process still involves production within living cells, biosimilars undergo fewer clinical trials than do their reference biologics. This ultimately reduces the cost of production and the cost of the biosimilar drug compared to its reference biologic. Currently, seven biosimilars have been approved by the United States Food and Drug Administration (FDA) for use in Crohn's disease, ulcerative colitis, and colorectal cancer. There are other biologics involved in treating gastroenterologic diseases for which there are no FDA approved biosimilars. Although biosimilars have the potential to reduce healthcare costs in chronic disease management, they face challenges in establishing a significant market share. Physician comfort in prescribing reference biologics instead of biosimilars and patient reluctance to switch from a biologic to a biosimilar are two common contributing factors to biosimilars' slow increase in use. More time will be needed for biosimilars to establish a larger and more consistent market share compared to their reference biologics. Additional da-

ta confirming the safety and efficacy of biosimilars, increased number of available biosimilars, and further cost reduction of biosimilars will all be necessary to improve physician confidence in biosimilars and patient comfort with biosimilars.

Key words: Biosimilars; Inflammatory bowel disease; Biologics; Inflammation; Drug class

Core tip: This study elucidates the unique properties of biosimilars as a drug class and their effectiveness for inflammatory bowel conditions in lieu of first line biologics.

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INTRODUCTION

Therapeutic proteins, also known as biologics, are pharmaceutical agents created in a laboratory setting to mimic the structure of naturally produced proteins in the body. They may either mimic the natural protein's function or antagonize the function of the natural protein. These drugs are produced in living cellular systems, and they have proven to be effective treatment for many diseases including rheumatoid arthritis, ankylosing spondylitis, and inflammatory bowel diseases[1]. Unfortunately, the high costs of therapeutic protein place a heavy financial burden on the healthcare system and limit the number of patients that are able to be covered. For example, monoclonal antibody therapy - one type of therapeutic protein - is projected to reach $125 billion in global sales by 2020[2]. As patents on biologic drugs expired, biosimilar drugs were developed and are helping to address this growing issue. The United States Food and Drug Administration (FDA) defines a biosimilar as "a biological product that is highly similar to and has no clinically meaningful differences from an existing FDA-approved reference product." These drugs are still created using living cells, but the synthesis pathway of the reference biologic is proprietary. Biosimilar developers instead analyze the final biologic and attempt to reverse engineer a feasible synthesis pathway.

The Affordable Care Act created a more efficient licensing pathway for these biosimilar drugs provided it can be proven that the biosimilar drug is not significantly different from its reference product in terms of effectiveness or safety. The process for biosimilar approval in Europe was established prior to that of the United States. The European Medicine Agency (EMEA) and the associated Committee for Medicinal Products for Human Use (CHMP) evaluate data gathered by pharmaceutical companies seeking approval for prospective biosimilars[3]. In both the United States and in Europe, biosimilar drugs must undergo structural analyses, functional assays, animal studies, and finally clinical studies. Throughout each step of the abbreviated approval process the biosimilar drug is compared to its reference biologic and assessed for similarity[4]. In contrast, a standard biological product undergoes a more traditional set of trials involving laboratory and animal testing to determine safety in humans followed by clinical trials.

A study in Europe examining the acceptance of biosimilars found that very few patients were willing to switch to a biosimilar if they were already taking a biologic. Increases in prevalence of biosimilar treatment are driven primarily by new patients that start on a biosimilar first. Even in a new patient population, significant price reductions, sometimes 50% or more, must be in place for physicians to consider prescribing a biosimilar[5]. Market shares for biosimilars are increasing slowly. For example, the filgrastim biosimilar Zarxio held 15% of the United States filgrastim market in 2016 and the infliximab biosimilar Inflectra held less than 10% of the infliximab market (United States biosimilar market). Gastroenterology has many potential benefits from biosimilars in terms of increasing treatment access while reducing treatment costs. Inflammatory bowel diseases and gastrointestinal cancers utilize biologics regularly. Within the endocrinological function of gastroenterology, biosimilar insulin is also an area of active investigation as insulin costs and prevalence of diabetes both continue to increase.

INFLAMMATORY BOWEL DISEASES

The anti-tumor necrosis factor alpha (TNF-α) biologic infliximab is an effective treatment for inflammatory bowel diseases. The PLANETAS study, a phase Ⅰ study, established biosimilar infliximab, CT-P13, as having equivalent pharmacokinetics with comparable safety and efficacy profiles to its reference infliximab while the PLANETRA study, a phase Ⅲ study, found that CT-P13 had equivalent efficacy to reference infliximab after 30 wk of treatment[6,7]. The patient populations in these studies, however, were patients with ankylosing spondylitis and rheumatoid arthritis. The PROSIT-BIO cohort study specifically investigated the safety and efficacy of CT-P13 in patients with ulcerative colitis and Crohn's disease. The data showed comparable results to those of similar studies with reference infliximab, but the study did not directly compare the biosimilar with its reference biologic[8]. A prospective study of 210 patients also found that CT-P13 is effective in inducing clinical remission in Crohn's disease and ulcerative colitis but noted decreased response to treatment and increased risk of allergic

reactions in those previously treated with reference infliximab[9]. A study of 96 patients comparing the efficacy of infliximab compared to biosimilar CT-P13 in maintaining remission in inflammatory bowel diseases found similar long-term outcomes and safety between the two treatment groups[10]. Additionally, a study on CT-P13 in pediatric Crohn's disease reported remission after three doses in 24 of 36 patients and clinical response in 31 of 36[11]. CT-P13 is currently marketed as Remsima™ and Inflectra™.

A double-blind, parallel-group study comparing another infliximab biosimilar, SB2, with reference infliximab in 584 patients with rheumatoid arthritis demonstrated similar safety, efficacy, immunogenicity, and pharmacokinetics at weeks 30 and 54[12,13]. SB2 is currently marketed as Flixabi® and approved for treatment of multiple chronic inflammatory diseases including the treatment of Crohn's disease and ulcerative colitis in patients between the ages of 6 and 17.

A 2016 study examined survey responses of inflammatory bowel disease specialists regarding biosimilars. Out of 118 responses, only 19.5% were not confident with using biosimilars, and 44.4% believed the biosimilar to be interchangeable with the reference biologic. The primary perceived benefit reported was cost reduction, and the main concern was immunogenicity. A prospective multicenter study done in 2015 similarly elucidates a positive response profile of biosimilars, and further illustrates safety regarding immunogenicity[9]. The overall positive outcomes when comparing biosimilar infliximab to its reference biologic have improved physicians' attitudes towards biosimilars in the context of treating inflammatory bowel disease.

INTERCHANGEABILITY

In addition to biosimilars, there exist "interchangeable" products. In order for a biosimilar to be considered interchangeable, it must undergo additional testing. The biosimilar in question must have equal clinical efficacy as its reference product and there must be no changes in safety or efficacy when switching between the biosimilar and its reference product. The purpose of a biosimilar being proven to be interchangeable is that the biosimilar may then be substituted in place of its reference biologic without physician involvement[14]. The risks and concerns involved with this substitution are that switching from a reference biologic to a biosimilar may have reduced efficacy or increased immunogenicity. Data that display similar efficacy, safety profiles, and immunogenicity between a biosimilar and its reference product are not sufficient to determine the effects of switching between the products. While the Canadian Agency for Drugs and Technologies in Health (CADTH) has reported equivalent safety and efficacy in switching from reference biologic to biosimilar in treatment of rheumatologic diseases, the one cohort study examining interchangeability in treatment of Crohn's disease and ulcerative colitis had a sample size of eight patients at week 48 following the change to biosimilar infliximab[15]. Six of the eight patients continued in remission, but the small sample size causes difficulty in extrapolating the findings to the general population[16]. The NOR-SWITCH trial examined the safety and efficacy of switching from reference infliximab to a biosimilar infliximab compared to keeping patients on the reference infliximab. The study was constructed as a non-inferiority study and included patients with six different chronic inflammatory diseases. The trial concluded that switching to biosimilar infliximab was not inferior to continuing reference infliximab[17]. While the study provides a necessary foundation for interchangeability studies, it did not control for variables within the patient population and it did not study each disease individually. A prospective study of 133 patients with inflammatory bowel disease measured antibodies to infliximab as well as C-reactive protein and erythrocyte sedimentation rate in context of disease activity scores to obtain numerical measurements of interchangeability. It found no differences between reference infliximab and biosimilar infliximab, but it also did not directly compare to continuing patients on reference infliximab[18]. A study investigating efficacy, pharmacokinetics, and immunogenicity when switching from reference infliximab to a biosimilar infliximab in pediatric patients with inflammatory bowel disease demonstrated no significant differences compared to continuing therapy with reference infliximab[19]. Additional studies focused on specific diseases and patient populations in the future will continue to advance biosimilars to interchangeable products.

LIMITATIONS

The main concerns raised regarding biosimilars are immunogenicity, efficacy, adverse effects when switching from a biologic to a biosimilar, and possible long-term effects[20]. This issue has been well documented in two recent 2017 trials, comparing the implications of switching from an infliximab innovator to biosimilar, over the span of 1 year in IBD patients. With its results showing enhanced clinical effectiveness and an appropriated side effect profile[16,18]. FDA approval addresses questions regarding immunogenicity and efficacy. Although the approval process for biosimilars is expedited, potential biosimilars must prove equivalent efficacy without additional immunogenicity or side effects[21,22]. In terms of switching between products, studies have shown that switching between two structurally different proteins that have a similar intended effect is not associated with increased risk for adverse events[23]. Thus, switching between proteins that share a nearly identical structure should also present no additional risk. The concern that has yet to be addressed is the potential long-term effects. As all other characteristics of biosimilars are comparable to biologics, it seems unlikely that long term ris-

Table 1 Biologics approved for the management of gastrointestinal inflammatory and oncological conditions

Condition/disease	Biologic	FDA approved biosimilar
Crohn's disease	Infliximab (anti-TNFα)	Remsima, Inflectra, Renflexis, Flixabi
	Adalimumab (anti-TNFα)	Amjevita, Cyltezo
	Certolizumab (anti-TNFα)	NA
	Vedolizumab (anti-α4-integrin)	NA
	Natalizumab (anti-α4-integrin)	NA
	Ustekinumab (anti-IL-antibody)	NA
Ulcerative colitis	Infliximab (anti-TNFα)	Remsima, Inflectra, Renflexis, Flixabi
	Adalimumab (anti-TNFα)	Amjevita, Cyltezo
	Golimumab (anti-TNFα)	NA
Colorectal cancer	Bevacizumab (anti-VEGF)	Mvasi
	Ramucirumab (anti-VEGF)	NA
	Cetuximab (anti-EGFR)	NA
	Pantimumab (anti-EGFR)	NA
Gastric cancer	Trastuzumab (anti-HER2/neu)	Ogivri

VEGF: Vascular endothelial growth factor; IL: Interleukin; EGFR: Epidermal growth factor receptor; NA: An FDA approved biosimilar is not available.

ks would be substantially different. However, only more time and more data will be able to answer with certainty. The main limitation of biosimilars is patient and physician acceptance with many patients preferring to stay on biologics and physicians preferring to prescribe biologics.

FUTURE DIRECTIONS

Continuing to manufacture new biosimilars as patents on biologics expire will be the primary means of increasing biosimilar prevalence. Many biologics used to treat inflammatory bowel disease or gastrointestinal cancers do not have corresponding biosimilars at this time (Table 1). Overall acceptance of biosimilars of patients will depend on comfort of physicians educating patients and prescribing biosimilars. Physician comfort will depend on additional clinical trials and increasing the amount of available data. Improving insurance coverage of new biosimilars will also increase patient access to biosimilars. Insurance companies are more comfortable covering biosimilars that have been on the market longer, but as more biosimilars become available it is possible that they will provide coverage even for new biosimilars.

DISCUSSION

Currently there are seven biosimilars approved in the United States. The most recent, biosimilar bevacizumab, was approved in September, 2017. In the case of infliximab and its biosimilar, it is likely that greater price differences will have to be seen before physicians will be convinced to switch away from the reference product. The average cost per year for infliximab treatment as of 2012 was $24000[22]. As of 2016, there was only a 15% price difference between infliximab and its biosimilar. The reluctance of both patients and physicians to switch to a biosimilar may imply that increases in market shares for biosimilars will be a matter of time as more biologic-naïve patients are placed on biosimilars to begin their treatment regimen. The reluctance of physicians also may affect clinical trials and even patient outcomes through the nocebo effect, which has been documented as causing generalized side effects despite a lack of plausible pharmacological mechanism based on the drug itself or side effects more severe than observed when medication use is blinded[24,25]. The way in which a physician discusses the effects of a drug with a patient influence the possibility of a nocebo effect. As such, patients receiving biosimilars from physicians who are reluctant to prescribe them may experience more adverse events or decreased treatment efficacy.

Additional studies will also be needed to further examine interchangeability of biologics and biosimilars. The case of switching from a biosimilar to a biologic if the biosimilar does not produce significant clinical improvement should also be explored, especially considering the number of biologic-naïve patients who may be started on a biosimilar rather than biologic therapy. However, as illustrated above, numerous studies have shown to carry similar efficacy when switching from an original biologic agent to a biosimilar. Biosimilars have great potential to improve access to disease modifying therapies over a wide range of chronic illnesses, extending even to some cancers. The more cost-efficient manufacturing process of biosimilars may also open the way to greater experimentation with pharmacological therapies.

CONCLUSION

Biosimilars have the potential to improve patient access to high level drug therapies as well as alleviate the financial strain that chronic illnesses place upon healthcare systems worldwide. To accomplish this, however, physicians will need to be more comfortable prescribing biosimilars instead of their reference products and the prices of biosimilars will need to be significantly lower than their biological counterparts.

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REVIEW

PNPLA3 rs738409 underlies treatment response in nonalcoholic fatty liver disease

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Abstract

Non-alcoholic fatty liver disease (NAFLD) has now become the leading cause of chronic liver disease with its growing incidence worldwide. Patatin-like phospholipase domain-containing protein 3 (PNPLA3) rs738409 C > G reflects one of the critical genetic factors that confers high-risk to NAFLD. However, the role of PNPLA3 polymorphism in NAFLD treatment remains uncertain. Here, the present review reveals that NAFLD patients with G-allele at PNPLA3 rs738409 (PNPLA3 148M variant) are sensitive to therapies of lifestyle modification, dipeptidyl peptidase-4 inhibitors, and bariatric surgery. They exhibit much significant reduction of liver fat content, in concurrence with weigh loss and abolished insulin resistance, as compared to those of C-allele carriers. In contrast, patients bearing PNPLA3 rs738409 C-allele (PNPLA3 148I variant), instead of G-allele, demonstrate greater beneficial effects by omega-3 poly-unsaturated fatty acids and statin intervention. Improved adipose tissue-liver interaction and decrease in intrahepatic triglyceride efflux may contribute to the PNPLA3 rs738409 related diversities in therapeutic efficacy. Therefore, PNPLA3 rs738409 underlies the response to a variety of treatments, which warrants a personalized, precise medicine in NAFLD on the basis of genotype stratification.

Key words: Non-alcoholic fatty liver disease; Patatin-like phospholipase domain-containing protein 3; Treatment; Lifestyle; Pharmacotherapy; Surgery; Polymorphism

Core tip: Patatin-like phospholipase domain-containing protein 3 (PNPLA3) rs738409 imposes universal, yet distinctly different, impact on various therapies in nonalcoholic fatty liver disease (NAFLD) patients. As compared to those with C-allele, patients with PNPLA3 rs738409 G-allele (PNPLA3 148M variant) show grater improvement in response to lifestyle modification, dipeptidyl peptidase-4 inhibitor ingestion, and bariatric surgery. In contrast, NAFLD patients carrying PNPLA3 rs738409 C-allele (PNPLA3 148I variant) are much sensitive to both omega-3 poly-unsaturated fatty acids and statin intervention. These diversities in treatment response warrant a personalized, precise medicine in NAFLD by stratification of PNPLA3 rs738409 genotype.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), a metabolic stress-induced chronic liver disease, has undergone a dramatic increase in prevalence worldwide over the last few decades with a morbidity rate of 15%-40%[1,2]. Over 20% of NAFLD patients progress from simple steatosis to non-alcoholic steatohepatitis (NASH)[3,4], which has a >10% chance of developing into liver fibrosis/cirrhosis and even hepatocellular carcinoma[5]. Due to the considerable burden of NAFLD on public health, it has become an emergent target for clinical intervention.

In addition to a sedentary lifestyle and Western diet, genetic polymorphism of various genes is considered as the other important factor in NAFLD predisposition[6]. In a genome-wide association scan (GWAS) of nonsynonymous sequence variations (n = 9229), patatin-like phospholipase domain-containing protein 3 (PNPLA3) rs738409 C > G (PNPLA3 I148M) was identified as a risk factor for NAFLD in Hispanic, African American and European Americans[7]. Further studies in multiple ethnic populations confirmed the effect of PNPLA3 I148M on NAFLD susceptibility, with a spectrum ranging from steatosis, NASH, to liver fibrosis[8-11]. PNPLA3 encodes the adiponutrin which is sited in the endoplasmic reticulum and on lipid droplets in hepatocytes. Possessing a patatin-like domain at the N-terminal, PNPLA3 shows hydrolase activity against glycerolipids (triacylglycerol, diacylglycerol, and monoacylglycerol), and has a crucial role in the homeostasis of lipid metabolism[12,13]. However, PNPLA3 148M functions in a "loss-of-function" way and leads to low levels of glycerolipid hydrolysis in the liver and inhibition of lipid outflow to peripheral adipose tissues[12,13]. Therefore, the PNPLA3 148M variant contributes to hepatic steatosis and related disorders depending on its interference with lipometabolic balance.

Current therapeutic approaches for NAFLD include lifestyle modification (*e.g.*, diet therapy and physical activity)[14,15], pharmacotherapy [*e.g.*, omega-3 fatty acids, statins, and dipeptidyl peptidase-4 (DPP-4) inhibitors][16], and bariatric surgery (*e.g.*, nonadjustable or adjustable banding, vertical banded gastroplasty, and gastric bypass)[17]. It is rational to propose that PNPLA3 rs738409 C > G (PNPLA3 148M) may affect the efficacy of NAFLD therapy due to the disturbance of glycerolipid-metabolic homeostasis. In this review, we summarize the therapeutic outcomes in NAFLD patients with different genetic backgrounds in order to highlight the interaction between PNPLA3 genotypes and treatment response.

LIFESTYLE MODIFICATION

Lifestyle modification, including a hypocaloric diet and/or increased physical activity, has been recommended as first-line therapy by the Diagnosis and Management of NAFLD practice guideline[18]. To test the effects of PNPLA3 polymorphism on treatment response to lifestyle modification, a randomized controlled trial was conducted in 154 adult Hong Kong residents with NAFLD[19]. Following equal randomization into the intervention and control group, respectively, 77 NAFLD patients received dietary consultation sessions that encouraged an individual-designed menu with emphasis on fruit and vegetables, and moderate-carbohydrate, low-fat, low-glycemic index, and low-calorific products in appropriate portions according to the recommendations of the American Dietetic Association. In addition, the patients were instructed to develop a routine exercise habit (30 min every day). Evaluation of these patients using proton magnetic resonance spectroscopy (^{1}H-MRS) showed that NAFLD patients carrying the G-allele at PNPLA3 rs738409 demonstrated a greater reduction in intrahepatic triglyceride content (IHTG) (GG: 11.3 ± 8.8%) compared to those with the C-allele (CC: 3.7 ± 5.2%, CG: 6.5 ± 3.6%) at the end of the 12-mo treatment. A greater decrease in body weight, waist-to-hip ratio (WHR), total cholesterol (TC), and low-density lipoprotein (LDL) cholesterol was also confirmed in the G-allele, but not the C-allele carriers with the exception of biochemical and liver stiffness measurements. The reduction in hepatic fat was parallel with the decrease in body weight and improvement in LDL cholesterol and TC. Multivariate analysis showed that PNPLA3 genotype and body mass index (BMI) change were the only factors correlated with IHTG reduction in the intervention group. In contrast, no correlations between PNPLA3 rs738409 and changes in IHTG or other measured parameters were found in the control group.

Eight subjects with a homozygous PNPLA3 rs738409

G-allele (PNPLA3-148MM) and 10 with a homozygous PNPLA3 rs738409 C-allele (PNPLA3-148II) were recruited for a further 6-d trial of a hypocaloric (1000-kcal deficit/d), low-carbohydrate diet (< 30 g/d)[20]. Despite a similar percentage of liver fat as shown by 1H-MRS on day 0, the PNPLA3-148MM group experienced a significantly greater reduction in liver fat than the PNPLA3-148II group (day 6, PNPLA3-148MM *vs* PNPLA3-148II: 10.2 ± 1.8% *vs* 11.9 ± 2.1%) independent of a comparable weight loss. In this study, no statistical differences were found in plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ-glutamyltransferase (GGT), and free fatty acid (FFA) concentrations between the two groups. It is necessary for individuals with NAFLD to achieve amelioration of steatosis by a 3%-5% weight loss, and to achieve an improvement in necroinflammation of up to 10%[18, 21]. Thus, the incomplete response to diet therapy may be due to an inadequate weight reduction (-3.7 ± 0.5% in the PNPLA3-148MM group, -3.3 ± 0.3% in the PNPLA3-148II group). In addition, 143 Caucasian Polish patients with NAFLD were prospectively enrolled in a dietary intervention[22]. All overweight or obese individuals received a 500 kcal restriction diet, whereas patients with normal weight were permitted a dietary intake that was consistent with physiological needs. The total fat content, including mono- and polyunsaturated fats, was reduced to an energy intake of 25%. Additionally, daily cholesterol consumption was less than 300 mg. After 4 mo of the intervention, individuals with the MM genotype of PNPLA3 exhibited a greater improvement in WHR compared to those with the Ⅱ genotype. In support of the close correlation between WHR and hepatic steatosis[23], decreased WHR facilitates the amelioration of NAFLD on the basis of attenuated abdominal obesity.

Peripheral lipolysis has been identified as the major source of intrahepatocellular triglycerides[24,25], one of the dominant lipid components responsible for hepatic steatosis. Based on the significant correlation between extrahepatic lipolysis and the change in liver fat content[24], the decrease in liver fat following lifestyle modification is attributed to a change in peripheral lipolysis and then FFA delivery to the liver. Using [$^2H_5$] glycerol, whole-body lipolysis can be analyzed by the rate of appearance (Ra) of glycerol[20]. Enhanced percentage suppression of glycerol Ra increased the anti-lipolytic effect of insulin by the ketogenic diet[20]. PNPLA3-148MM, but not PNPLA3-148II, significantly promoted the suppression of glycerol Ra (37 ± 5% before and 51 ± 4% after the ketogenic diet)[20]. These findings suggest that a greater improvement in the insulin sensitivity of individuals with PNPLA3 148MM compared to those with PNPLA3 148II could have contributed to the greater reduction in liver fat following lifestyle modification.

PHARMACOTHERAPY

NAFLD, with the hallmark of excessive triglyceride accumulation, is considered the hepatic manifestation of the metabolic syndrome (MetS). The co-existence of other MetS components (*e.g.*, dysglycemia, decreased HDL cholesterol and arterial hypertension[26]) can also be risk factors for NAFLD severity[27]. Thus, medications associated with glycolipid metabolism (*e.g.*, omega-3 fatty acids, statins, and DPP-4 inhibitors) have been used in the management of NAFLD-related hepatic disorders ranging from steatosis and steatohepatitis to liver fibrosis.

Omega-3 fatty acids

One hundred and three subjects from six hospitals in the south of England were enrolled in a multicenter, double-blind, placebo-controlled clinical trial, "the WELCOME trial", which was performed to test the polymorphism-based therapeutic effects of high-dose omega-3 fatty acids on NAFLD[28]. The primary outcomes were a decrease in percentage liver fat and improvement in liver fibrosis scores. A total of 51 participants block randomized to omega-3 fatty acid ethyl esters, received Omacor® 4 g/d [4 × 1000-mg capsules of 460 mg eicosapentaenoic acid (EPA) and 380 mg docosahexaenoic acid (DHA)] for 15-18 mo. The remaining 52 NAFLD patients were treated with a placebo of isocaloric olive oil (4 g/d) containing approximately 67% oleic acid, 15% linoleic acid, 15% palmitic acid, 2% stearic acid, and 1% alpha linolenic acid. According to the 1H-MRS results, both PNPLA3 148I/I and 148I/M carriers demonstrated an adjusted mean decrease in liver fat percentage (148I/I: -7.05%, 148I/M: -7.30%) following the DHA+EPA intervention, whereas the PNPLA3 148M/M group had a slight increase in liver fat percentage (2.75%). Moreover, regression modeling demonstrated that the PNPLA3 148 M/M genotype was independently associated with the end-of-study liver fat percentage.

Similar findings were obtained in a randomized controlled trial of DHA supplementation (250 mg/d or 500 mg/d) in obese Italian children with ultrasound-diagnosed NAFLD[29]. As assessed by the combined DHA 250 mg/d and 500 mg/d groups versus placebo group, the 24-mo DHA trial resulted in a decreased probability of severe liver steatosis, with an independent effect of PNPLA3 polymorphism on the response to DHA. Somers' D model of liver fat evaluation revealed that the 148M allele of PNPLA3 predisposes carriers to low treatment response, with a 50% higher probability of more severe steatosis at the end of the trial. In contrast, a greater response to DHA was detected in those who were homozygous for the 148I allele in comparison with heterozygotes. An association between dietary N-6/N-3 polyunsaturated fatty acids (PUFA) ratio and hepatic fat content, and even serum ALT, was previously shown in multiethnic obese children who were homozygous for the 148M PNPLA3 allele[30]. The PNPLA3 148M/M genotype minimizes the secretion of both large TG-rich very-low-density lipoprotein particles and apoB100, and partially decreases lipolytic activity and lysophosphatidic

acid acyl-CoA transferase activity[31]. Furthermore, it contributes to the distorted fatty acid composition of liver lipid droplets by facilitating the differential incorporation of various types of fatty acids[32,33]. As a result, human PNPLA3 I148M is thought to be responsible for the abnormal efflux and remodeling, but not the influx of hepatic lipid. Omega-3 PUFAs (N3-PUFAs), including EPA and DHA, exert their pharmaceutical activity on liver fat reduction mainly by inhibiting de novo lipogenesis through SREBP-1c and ChREBP downregulation[34]. Therefore, the PNPLA3 148M allele counteracts the benefit of N3-PUFAs treatment via the limited outflow of liver fat.

Statins

With the exception of PUFAs, the interaction between statin use and PNPLA3 genotype on the risk of NASH was investigated in a multicenter cohort (n = 107) of European descent from Italy and Finland[35]. Each subject underwent liver biopsy due to increased liver enzymes, ultrasonographic evidence of steatosis and risk factors, or routine examination during bariatric surgery. Following different types and different intensities of treatment (49% on simvastatin, 27% on rosuvastatin, 17% on atorvastatin, 4% on pravastatin, and 2% on fluvastatin; 15% on high-intensity, 73% on moderate-intensity, and 12% on low-intensity treatment), statins demonstrated dose-dependent protective effect on steatosis, steatohepatitis, and liver fibrosis for at least 6 mo. In support of the findings in the N3-PUFAs intervention, individuals carrying PNPLA3 I148M alleles were susceptible to the full spectrum of liver damage. Statin use was negatively associated with steatohepatitis in patients without PNPLA3 148M variant diagnosed with NAFLD activity score. NASH is characterized by the excessive accumulation of hepatic free cholesterol on the basis of activated 3-hydroxy-3-methyl-glutaryl coenzyme-A reductase (HMGCR), which acts as the rate-limiting enzyme in cholesterol biosynthesis[36-38]. As HMGCR inhibitors, statins have been linked to a reduced risk of NAFLD in epidemiological studies[39]. Therefore, down-regulation of cholesterol synthesis is thought to underlie the therapeutic effects of statins on NAFLD/NASH. In contrast, PNPLA3 I148M inhibits lipid efflux to abolish the statin-dependent decrease in the hepatic cholesterol pool. This may provide a rational explanation for the blunted benefit of statin treatment in patients with the PNPLA3 148M allele.

DPP-4 inhibitors

A 33.1-mo study determined the efficacy of alogliptin (25 mg/d), a selective DPP-4 inhibitor, in 41 biopsy-proven Japanese NAFLD patients with type 2 diabetes mellitus (DM)[40]. Of the metabolic and biochemical parameters measured, patients with the G-allele at PNPLA3 rs738409 (genotype CG/GG) showed a positive correlation between improvements in hemoglobin A1c (ΔHbA1c) levels and changes in aminotransferases (ΔALT and ΔAST). It is also worth noting that patients with the CG/GG genotype, instead of the CC genotype, exhibited significantly greater improvements in TC, TG and hyaluronic acid after their intentional weight loss. As effective medications for glucose metabolism, DPP-4 inhibitors (*i.e.*, sitagliptin, alogliptin) have a beneficial impact on HbA1c which depends on the prolonged half-life of glucagon-like peptide 1[41,42]. The serum level of ALT has been proved to be significantly correlated with increased fasting plasma glucose (FPG), a consequence of abnormal HbA1c[43]. The decrease in HbA1c following DPP-4 inhibitor treatment, especially in patients with high HbA1c ($\geqslant$ 7.5%), is positively associated with amelioration of AST and ALT levels[44]. Thus, DPP-4 inhibitors are thought to prevent liver injury, including inflammation and hepatocyte ballooning in NAFLD patients with glucolipid dysmetabolism[45,46].

BARIATRIC SURGERY

Bariatric surgery is currently recommended in NAFLD patients with a BMI $\geqslant$ 40 kg/m^2, or BMI $\geqslant$ 35 kg/m^2 with comorbidities and limited efficacy of diet therapy and/or physical activity[47-50]. In addition to its effect on weight loss[51,52] and metabolic parameters[53,54], bariatric surgery has been demonstrated to improve the pathologic features of NAFLD[55,56]. Notably, the PNPLA3 polymorphism has an influence on the response of individuals to therapy.

Bariatric surgery, including gastric bypass surgery in 43 individuals and gastric sleeve surgery in 41, was performed in obese adult Caucasian subjects (BMI 35-64 kg/m^2)[57]. Compared with that at baseline, carriers of the PNPLA3 I148M allele M exhibited a higher median weight loss (47 kg, 36.1% of total) than those with genotype Ⅱ (38 kg, 32.3% of total) 12 mo after bariatric surgery. Consistent with this weight loss, the magnetic resonance imaging-based estimation of the Folch value[58] revealed a significantly higher decrease in liver fat in patients carrying allele M rather than allele Ⅰ, with up to 65.5%, 70.7%, and 85.5% of their initial fat content in the Ⅱ, IM and MM genotypes, respectively. Fifty-seven percent of the study population was diagnosed with moderate-to-severe hepatic steatosis (grade 2 or 3) before surgery[57], and the improvements in body weight and liver fat led to a diminished percentage of patients with steatosis at the end point. Patients with the MM genotype showed a percentage change higher than those with the Ⅱ genotype. Multivariate regression analysis further indicated that the PNPLA3 polymorphism and the initial grade of steatosis were the strongest independent predictors of surgical efficacy in non-alcoholic fatty liver. The PNPLA3 polymorphism had no effect on serum ALT activity, which may be attributed to the recruitment of patients in the early stages of NAFLD.

The prospective, controlled Swedish Obese Subjects study, "the SOS study", enrolled individuals (men: BMI $\geqslant$ 34, women: $\geqslant$ 38) for bariatric surgery (surgery

Table 1 Effect of Patatin-like phospholipase domain-containing protein 3 rs738409 C > G p.I148M on therapeutic response in nonalcoholic fatty liver disease

Study	Country	Duration	Number	Intervention	Results
Sevastianova *et al*[20] (2011)	Finland, Italy	6 d	18 (all in intervention group)	Hypocaloric (1000-kcal deficit/d), low-carbohydrate diet (< 30 g/d)	Δliver fat[a]
Shen *et al*[19] (2015)	China	12 mo	Intervention group (n = 77), control group (n = 77)	Exercise and limitation on caloric intake	ΔIHTG[d], ΔBody weight[c], ΔWC[c], ΔWHR[c], ΔTC[c], ΔHDL-C[d]
Milkiewicz *et al*[22] (2016)	Poland	4 mo	323 (143 in intervention group; 180 in control group)	Diet with 500 kcal resriction for subjects (BMI > 25); maintance of body weight for subjects (BMI ⩽ 25)	ΔWHR[c]
Scorletti *et al*[28] (2015)	United States	15-18 mo	Omacor®: n = 51; placebo: n = 52	Omacor®, 4 g/d (460 mg EPA + 380 mg DHA for 1 g Omacor)	Δliver fat[e]
Nobili *et al*[29] (2013)	Italy	24 mo	DHA 250 mg/d (n = 20), DHA 500 mg/d (n = 20), placebo group (n = 20)	DHA 250 mg/d, DHA 500 mg/d, or placebo	Severe steatosis[g,h], TG[i] ALT[i]
Dongiovanni *et al*[35] (2015)	Italy, Finland	6 mo	107 (all in intervention group)	Drug: simvastatin (49%), rosuvastatin (27%), atorvastatin (17%), pravastatin (4%), fluvastatin (2%) Intensity: high (15%), moderate (73%), low (12%)	Risk of NASH[k]
Kan *et al*[40] (2016)	Japan	33.1 mo	41 (all in alogliptin group)	Alogloptin, 25 mg/d	ΔALT, ΔAST, ΔHbA1c[m]
Krawczyk *et al*[57] (2016)	Spain	12 mo	84 (all in surgery group)	Gastic bypass surgery; gastric sleeve surgery	Δweight[p], Δliver fat[o]
Palmer *et al*[59] (2012)	Sweden	2 yr; 10 yr	3473 (2nd year: 1624 in surgery group; 10th year: 1355 in surgery group)	nonadjustable or adjustable banding; vertical banded gastroplasty; gastric bypass	ΔHOMA-IR[q], Δserum TG[q], ΔALT[s]

[a]P < 0.05, PNPLA3 148M/M *vs* PNPLA3 148I/I; [c]P < 0.05, [d]P < 0.01, among PNPLA3 148I/I, 148I/M, and 148M/M in intervention group; [e]P < 0.05 PNPLA3 148I/I +148I/M *vs* PNPLA3 148M/M; [h]P < 0.001, PNPLA3 148M/M *vs* PNPLA3 148 I/M and [g]P < 0.05, PNPLA3 148I/M *vs* PNPLA3 148 I/I; [i]P < 0.05, PNPLA3 148M/M *vs* 148I/M; [k]P < 0.001, PNPLA3 148I/I *vs* 148I/M or PNPLA3 148I/I *vs* 148M/M; [m]ΔHbA1c correlates to ΔALT (P = 0.001) and ΔAST (P = 0.014) in patients with PNPLA3 rs738409 C/G+G/G; [o]P < 0.05, [p]P < 0.01, PNPLA3 148I/I *vs* PNPLA3 148I/M+148M/M; [q]P < 0.05, among PNPLA3 148I/I, 148I/M, and 148M/M in surgery group (2nd, 10th year); [s]P < 0.001, among PNPLA3 148I/I, 148I/M, and 148M/M in surgery group (2nd year). ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; IHTG: Intrahepatic triglyceride content; HbA1c: Hemoglobin A1c; HDL-C: High-density lipoprotein-cholesterol; HOMA-IR: Homeostasis model assessment-insulin resistance; TC: Total cholesterol; TG: Triglycerides; WC: Waist circumference; WHR: Waist-to-hip ratio.

group) or conventional treatment (matched control group)[59]. In contrast to the matched control group who underwent nonsurgical treatment (sophisticated lifestyle intervention and behavior modification), the surgery group received one surgical procedure (nonadjustable or adjustable banding, vertical banded gastroplasty, or gastric bypass) at baseline[60]. Weight losses of 25 ± 11%, 16 ± 11%, and 14 ± 14% were documented for gastric bypass, vertical-banded gastroplasty, and banding, respectively, after a 10-year follow-up[60]. PNPLA3 148M carriers showed a greater reduction in homeostasis model assessment-insulin resistance (HOMA-IR) and plasma ALT, together with a lower reduction in triglyceride levels, in comparison with PNPLA3 148I carriers. The prevalence of biopsy-proven hepatic steatosis increased up to 70% in an obese population (BMI ⩾30)[61], and even reached 91% in another ultrasound-based study[62]. Multivariate analysis provided deeper insight into the significant association between obese-related steatosis and impairment in both insulin sensitivity (*e.g.*, fasting insulin, HOMA-IR) and glucose metabolism (*e.g.*, FBG)[59]. Moreover, the occurrence of steatohepatitis markedly increased with grade of obesity, and was approximately 3% in the lean population, 19% in the obese population, and 50% in the morbidly obese population[63]. Augmentation of serum aminotransferases reflected hepatocyte injury with high sensitivity in these patients with steatohepatitis[64]. In contrast, according to the Diagnosis and Management of NAFLD guideline, weight reduction leads to the amelioration of NAFLD with steatosis resolution, improved HOMA-IR, and normalization of ALT[18].

An interesting common result in the two above-mentioned studies[57,59] was the association between PNPLA3 148M and some measured parameters (*e.g.*, liver fat content) which was abolished after weight loss induced by surgery. Marked weight reduction was achieved in both studies (over 30% weight loss after 12 mo in the former[57]; 20%-32% weight loss after 1 - 2 years and 14%-25% weight loss after 10 yr in the latter[60]), and no NAFLD occurred in the majority of the subjects at the end of the follow-up.

CONCLUSION

Taken together, these results show that PNPLA3 rs738409 has a universal, yet distinctly different, impact on the response to various therapies in NAFLD patients independent of age, gender, and ethnic background.

Although the G-allele at PNPLA3 rs738409 (PNPLA3 148M variant) is associated with more severe NAFLD than the C-allele (148I variant), it results in a greater reduction in liver fat following lifestyle modification, bariatric surgery, and pharmacotherapy with DPP-4 inhibitors. The concurrence of weight loss, improved systemic glycolipid metabolism (WHR, TC, TG, LDL, and HbA1c) and decreased intrahepatic fat content highlight an interaction between peripheral adipose tissue and the liver on the actions of the PNPLA3 polymorphism (Table 1). Enhanced systemic insulin sensitivity (*e.g.*, lowered HOMA-IR) with an anti-lipolytic effect and inhibition of periphery-to-liver delivery of FFAs are thought to underlie the benefits of the PNPLA3 rs738409 G-allele over the C-allele. Nevertheless, the hepatoprotective effect, with down-regulated biomarkers of plasma ALT, AST, ALP, and GGT, may not be necessary for the beneficial acquisition.

The risk allele of PNPLA3 rs738409 C > G (PNPLA3 I148M) predisposes NAFLD patients to a poor treatment response to pharmacotherapy (*e.g.*, N3-PUFAs and statins), with increased liver fat percentage and a higher probability of NASH, compared with wild-type PNPLA3 (PNPLA3 148I variant) (Table 1). Both N3-PUFAs, including EPA and DHA, and statins prevent de novo lipogenesis. However, the PNPLA3 148M allele inactivates hepatic glycerolipid hydrolysis due to its "loss-of-function" phenotype and minimizes lipid efflux from the liver to peripheral adipose tissues. This may result in a counteracting mechanism to limit the beneficial effect of pharmacotherapy.

NAFLD is well described to be a complex disease with polymorphic association to multiple genes[6]. Limited number of PNPLA3 variant (*e.g.*, rs738409) among these ones has a significant contribution, whereas variants in TM6SF2[65], MBOAT7[66] and GCKR[67] show the moderate-size effects. Besides, large number of variants in APOB[68], APOC3[69], LYPLAL1[70], MTTP[68], LPIN1[71], SOD2[72], UCP2[73], ENPP1[74], IRS1[74], IL28B[75], KLF6[76], MERTK[77], and Irisin[78] action in a low-effect manner. Effect of any risk variant of NAFLD is unlikely to be clinical meaningful. Nonetheless, the variant-dependent difference in treatment response provides prospect for the personalized risk algorithms and therapeutic strategy of NAFLD.

Given the close association of PNPLA3 polymorphism and NAFLD, patients with the G-allele at PNPLA3 rs738409 are thought to benefit from lifestyle modification, DPP-4 inhibitors, and bariatric surgery, which are characterized by weight loss and improved insulin resistance. NAFLD patients carrying the C-allele demonstrate sensitivity to N3-PUFAs and statin treatment. Therefore, stratification of the PNPLA3 rs738409 genotype may serve as a potential approach in the precise treatment of NAFLD.

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MINIREVIEWS

Risk factors for gastroesophageal reflux disease and analysis of genetic contributors

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Abstract

Gastroesophageal reflux disease (GERD) is a common gastrointestinal disorder with an increasing prevalence. GERD develops when the reflux of stomach contents causes troublesome typical and atypical symptoms and/or complications. Several risk factors of GERD have been identified and evaluated over the years, including a considerable amount of genetic factors. Multiple mechanisms are involved in the pathogenesis of GERD including: (1) motor abnormalities, such as impaired lower esophageal sphincter (LES) resting tone, transient LES relaxations, impaired esophageal acid clearance and delayed gastric emptying; and (2) anatomical factors, such as hiatal hernia and obesity. Genetic contribution seems to play a major role in GERD and GERD- related disorders development such Barrett's esophagus and esophageal adenocarcinoma. Twin and family studies have revealed an about 31% heritability of the disease. Numerous single-nucleotide polymorphisms in various genes like *FOXF1*, *MHC*, *CCND1*, anti-inflammatory cytokine and DNA repair genes have been strongly associated with increased GERD risk. GERD, Barrett's

esophagus and esophageal adenocarcinoma share several genetic loci. Despite GERD polygenic basis, specific genetic loci such as rs10419226 on chromosome 19, rs2687201 on chromosome 3, rs10852151 on chromosome 15 and rs520525 on the paired related homeobox 1 gene have been mentioned as potential risk factors. Further investigation on the risk genes may elucidate their exact function and role and demonstrate new therapeutic approaches to this increasingly common disease.

Key words: Single nucleotide polymorphisms; Genetic risk loci; Risk factors; Gastroesophageal reflux disease; Gastroesophageal reflux disease development

Core tip: Gastroesophageal reflux disease (GERD) is a common gastrointestinal disorder, which develops when the reflux of stomach contents causes troublesome symptoms and/or complications. Several risk factors of GERD have been identified and evaluated over the years. Motor esophageal and gastric abnormalities, along with anatomical factors could contribute to GERD development. Genetic contributors seem to play a major role in GERD. Numerous single-nucleotide polymorphisms in various genetic loci have been mentioned as potential risk factors. Further investigation on the risk genes may elucidate their exact function and role and demonstrate new therapeutic approaches to this increasingly common disease.

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is a highly frequent gastrointestinal disorder with prevalence up to 20% in Europe and USA. Its prevalence is also increasing in the Far East and other Asian areas[1,2]. According to the Montreal definition and classification system, GERD represents a condition which develops when the reflux of stomach contents causes troublesome symptoms and/or complications[3]. The disease is characterized by a broad spectrum of typical symptoms, such as heartburn and acid regurgitation, some atypical ones, like dysphagia and chest pain, in addition to extra-esophageal manifestations, such as asthma, chronic cough and laryngitis[4-6].

GERD and its complications have a high impact on every day clinical practice, as well as on patients suffering regularly from discomforting symptoms of refluxate[7]. Several risk factors of GERD have been identified and evaluated over the years, including a considerable amount of genetic factors[4,8].

A PubMed search was performed using the key words ("gastroesophageal reflux disease" OR "GERD" OR "chronic reflux disease" OR "reflux disease") AND ("oesophagus" OR "esophagus" OR "esophageal" OR "oesophageal") AND ("risk factors" OR "contributors") AND ("genetic background" OR "genetics" OR "susceptible genetic loci" OR " SNPs") AND ("Barrett's" OR "adenocarcinoma") AND ("genome wide association study" OR "GWAS") AND ("pathogenesis" OR "pathogenetic mechanisms") AND ("epidemiology") AND ("biomarkers"). The present review aims to report the genetic contributors of GERD, enriched with the pathogenetic mechanisms of the main risk factors, based on current literature.

PATHOGENETIC MECHANISMS OF THE MAIN RISK FACTORS

GERD is developed when detrimental to the esophagus factors transcend protective mechanisms, such as the esophago-gastric junction barrier and esophageal acid clearance, which normally contribute to the maintenance of a physiologically balanced condition. There are multiple mechanisms involved in the pathogenesis of GERD including: (1) motor abnormalities, such as impaired lower esophageal sphincter (LES) resting tone, transient LES relaxations (TLESR), impaired esophageal acid clearance and delayed gastric emptying; and (2) anatomical factors, such as hiatal hernia and obesity[4,9,10]. A valve mechanism exists between the esophagus and the stomach, formed by the LES and its adjacent anatomical structures, including the gastric sling and the crural diaphragm[11]. The main role of this valve mechanism in resting conditions is to create a fine-tuned high-pressure zone (15-30 mmHg above intragastric pressures), preventing gastric contents reflux. A minority of GERD patients experiences extremely low LES resting pressure (< 6 mmHg); every time stomach pressure exceeds the LES pressure, reflux occurs. Such a decreased LES resting tone is ordinarily correlated to severe grade of esophagitis and/or presence of GERD complications, including peptic stricture and Barrett's esophagus. However, in the majority of GERD patients, a high frequency of inappropriate LES relaxations is the cause of abnormal gastro-esophageal reflux[4]. TLESRs are spontaneous LES relaxations of 10-60 s duration, which are unrelated to swallowing[12,13]. Gastric distension, via stimulation of proximal gastric tension and stretch receptors, is considered the major contributor generating TLESRs. Although TLESRs occur in healthy individuals with a similar frequency to GERD patients, a higher percentage of TLESRs is associated with reflux in GERD patients[4,9,14-17]. Like LES resting pressure, the frequency of TLESRs is influenced by endogenous hormones (cholocystokinin, progesterone

etc.)[18], drugs (calcium channel blockers, nitrates, tricyclic antidepressant medications, benzodiazepine, anticholinergic drugs, theophylline *etc*.)[19], specific foods (fat, chocolate, *etc*.)[20] and daily habits (alcohol, caffeine, smoking)[21].

Ineffective esophageal motility (IEM) is considered, along with TLESR, another significant contributor to the appearance of GERD, as it leads to impaired esophageal clearance[22]. Esophageal acid clearance is a critical protective process involving primary and secondary peristalsis as well as the swallowing of salivary bicarbonate. Primary peristalsis occurs approximately 60 times per hour just after every swallow, whereas secondary peristalsis is observed in the absence of swallowing as a result of esophageal distension or of the presence of acidic contents into the esophageal lumen. The swallowing of saliva (pH 7.8-8.0) is pivotal in the accomplishment of esophageal acid clearance and in the restitution of esophageal pH. Evidence suggests that GERD patients show 2-3 fold longer acid clearance times compared to normal subjects[4]. The slower the esophageal clearance is, the longest the refluxate (comes into contact) with the esophageal mucosa. Thus, IEM leads to more severe GERD, in terms of both symptoms and mucosal damage[9].

Delayed gastric emptying might contribute to GERD in a small yet significant amount of patients, especially those who do not respond to proton pump inhibitors (PPIs) therapy[4,9]. An increase of the intra-gastric pressure, due to gastric distension, resulting in an overwhelming amount of refluxate, could be a putative mechanism for deteriorating GERD. Gastric distension could also contribute to an increase of the postprandial TLESR's rate[4].

Hiatal hernia is often found in patients with GERD with a prevalence of 0.8% up to 43.0%[23]. Hiatal hernia is considered to be a significant factor, since it disintegrates the gastro-esophageal sphincter, as the proximal stomach is dislocated into the chest and the crural diaphragm becomes separated from the LES. In patients with severe erosive esophagitis and in those with GERD complications, hiatal hernia is present in most cases. A linear correlation between hernia's size and the severity of reflux symptoms seems to exist. Hiatal hernia loosens the lower esophageal sphincter and increases the frequency of TLESRs. Moreover, it decreases esophageal clearance and enhances reflux by acting as a reservoir for gastric acid that becomes trapped in its sac[24,25].

Obesity has been considered to be a key risk factor of GERD. The rising rates of obesity (35.5% for men and 35.8% for women estimated by the National Health and Nutrition Examination Survey for the years 2005-2009)[26] are associated with early onset of GERD, as an independent factor (approximately 50% in morbid obesity)[27]. Among the possible mechanisms by which obesity promotes GERD, increased abdominal pressure, delayed gastric emptying, increased frequency of TLESR and reduced LES resting pressure are considered to play a crucial role[2,28,29]. The incidence of reflux symptoms rises progressively with increasing BMI. It is widely accepted that even short-term weight gain is associated with a three- to four-fold higher risk of GERD symptoms. This positive association between increasing BMI and GERD has been confirmed by a recent meta-analysis[29-31].

An interesting potential factor in peptic acid diseases is the gastric acid secretion in the interprandial periods. As suggested (1) by Feldman and Richardson[32] in a study on 8 patients with duodenal ulcer disease versus 7 normal subjects; and (2) by Caboclo *et al*[33] in a study on rats the possible mechanisms are: (1) increased oxyntic gland sensitivity; (2) hyperplasia of parietal cells, hypercorresponding to the vagal release of gastrin; or (3) cortical-stimulated secretion through methods of learning and memory, for example combing the food intake with specific sounds and emotions.

Additionally, several other factors have been asserted as causes of heartburn symptoms. Metabolic syndrome or its components- and especially hypertriglyceridemia- have been associated with erosive esophagitis or reflux symptoms respectively[34]. Zheng *et al*[21] found that a dose-dependent smoking was linked to the occurrence of gastroesophageal reflux symptoms with a risk of about 37% risk among women and 53% among men. In the same analysis, coffee intake was considered to be protective factor for GERD in men, contrary to women, probably due to different caffeine metabolic patterns. Exercise at work expedites the appearance of reflux symptoms, whereas leisure-time exercise was protective to the disease.

In a recently published study, Mungan and colleague[19] estimated the correlation between several categories of drugs and GERD. They deduced that non-steroidal anti-inflammatory drugs (especially when combined with acetylsalicylic acid, estrogen replacement therapy, calcium channel blockers (CCBs), nitrates, tricyclic antidepressant medications (particularly amitriptyline and clomipramine), hypnotics and benzodiazepine, anticholinergic drugs and theophylline promote the onset of GERD.

GENETIC RISK FACTORS

For over a decade, the role of genes in the development of gastroesophageal reflux disease and GERD-related disorders [Barrett's esophagus (BE), esophageal adenocarcinoma (EAC)] has been introduced. This statement was verified through population-based studies on twins. Numerous single-nucleotide polymorphisms (SNPs) have been proposed in genome-wide association studies (GWAS) as potential factors in the appearance of reflux disease[35,36] (Table 1).

Twin and family studies

In 2002, Cameron *et al*[37] examined 8411 pairs of twins [2178 monozygotic twins pairs (MZ) and 6233 dizygotic ones (DZ)] and concluded that the genetic influence on

Table 1 Studies performed on measuring the possible heritability of gastroesophageal reflux disease and the severity of reflux symptoms among twins and family members and on identifying the risk genetic loci for gastroesophageal reflux disease

Studies	
Twin and family studies	
Cameron A *et al*[37]	↑ Heritability of GERD, ↑ symptoms in MZ
Mohammed I *et al*[38]	↑ Heritability of GERD, ↑ symptoms in MZ
Reding-Bernal A *et al*[39]	↑GERD symptoms severity in families in Mexico
GERD risk genetic loci studies	
Ghoshal and Chourasia[8]	> 10 genes, up/down regulating GERD
Liu WF *et al*[40]	C allele in FOX1 rs9936833, A allele in MHC rs9257809 : ↑reflux symptoms
Gharahkhani P *et al*[41]	rs10419226 (chr 19), rs2687201 (chr 3) : ↑GERD symptoms
Bonfiglio F *et al*[35]	> 30 susceptible gene loci for GERD

GERD: Gastroesophageal reflux disease; MZ: Monozygotic twins; chr: Chromosome.

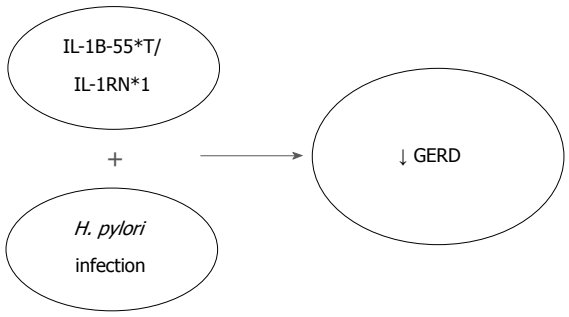

Figure 1 The presence of the genes IL-1B and IL-1RN combined with *Helicobacter pylori* infection is associated with hypochlorydria and thus reducing the risk for gastroesophageal reflux disease. GERD: Gastroesophageal reflux disease; *H. pylori*: *Helicobacter pylori*; IL-1B-511*T: Interleukin-1 beta T allele; IL-1RN: Gene encoding for a non-signaling molecule IL-1 receptor antagonist (IL-1Ra).

GERD reached 31% (95%CI, 0.23-0.38). After taking several factors into account, such as age, gender and daily habits, GERD was found to be strongly correlated to MZ. A year later, Mohammed *et al*[38] examined 4480 pairs of twins, which inferred 30% heritability of GERD, while the same symptoms in MZ outnumbered the DZ ones. Another study of 585 individuals concerning 32 families living in Mexico revealed an association between the severity of GERD symptoms, metabolic syndrome components and inflammatory markers due to common genetic back round[39].

Studies on identifying risk genetic loci for GERD

Ghoshal and Chourasia[8] attempted to enumerate the host genetic factors responsible for GERD and to explain their role in the pathogenesis of the disease, as well as its complications. The presence of the pro-inflammatory cytokines interleukin-1beta and IL-1RN (gene encoding for a non-signaling molecule IL-1 receptor antagonist) (IL-1B-511*T/IL-1RN*1) combined with *Helicobacter pylori* infection, has a protective effect against the development of GERD. Their presence results in extended gastritis and the destruction of parietal cells, leading in hypochlorhydria and thus reducing the risk for GERD (Figure 1). On the contrary, altered expression of Cyclooxygenase-2 (*COX-2*) (enzyme in prostaglandin biosynthesis), *IL-10* (anti-inflammatory cytokine), Glutathione-S-transferases (especially *GSTP1*b*), Cyclin D1 (*CCND1*) and DNA repair genes (*XRCC1, hMLH1*) have been associated with a high risk of GERD, BE or EAC. Additionally, the homozygous G/G variant genotype of epidermal growth factor (A61G), and the -C825T-genetic polymorphism of *GNB3* (G protein) also appear to contribute to an elevated risk of these conditions (Figure 2)[8].

In 2014, a study of 182 patients concluded that *FOXF1* (C allele in *FOXF1* rs9936833) (95%CI: 1.1-3.0; $P = 0.02$) and *MHC* (A allele in *MHC* rs9257809) polymorphisms (95%CI: 2.9-3.0; $P < 0.001$) were strongly associated with increased GERD risk in patients with reflux symptoms. *FOXF1* gene may play a role in the regulation of the contraction of the lower oesophageal sphincter, due to its involvement in the development of the gastrointestinal smooth muscle. Furthermore, the possibility that MHC genes are associated with HLA alleles and, therefore, could influence the activity of T-cells, reveals a T-cell involvement in reflux esophagitis (Figure 2)[40].

Gharahkhani *et al*[41] investigated the involvement of SNPs in the development of GERD and the shared genetic loci of GERD, BE and EAC.; The variability of SNPs could explain the 7% phenotypic variance present in GERD (BEACON and 23 and ME studies). Despite GERD polygenic basis, they suggested two specific genetic loci with high association with GERD: rs10419226 on chromosome 19 (95%CI: 1.00-1.07; $P = 0.038$) and rs2687201 on chromosome 3 (95%CI: 1.01-1.09; $P = 0.025$) (Figure 3).

Bonfiglio *et al*[35] recently conducted a GWAS meta-analysis of three independent population-based studies from Sweden, UK (TwinsUK) and Northern Finland (NFBC1966) in order to elucidate the pathogenesis of GERD. A total of 30 susceptible GERD risk loci were identified ($P < 0.5 \times 10^{-5}$). The strongest evidence suggesting a correlation between GERD and the various genetic loci was found at the SNP rs10852151 on chromosome 15 ($P = 2.3 \times 10^{-7}$) and at rs520525 on the paired related homeobox 1 (*PRRX1*) gene (*P*

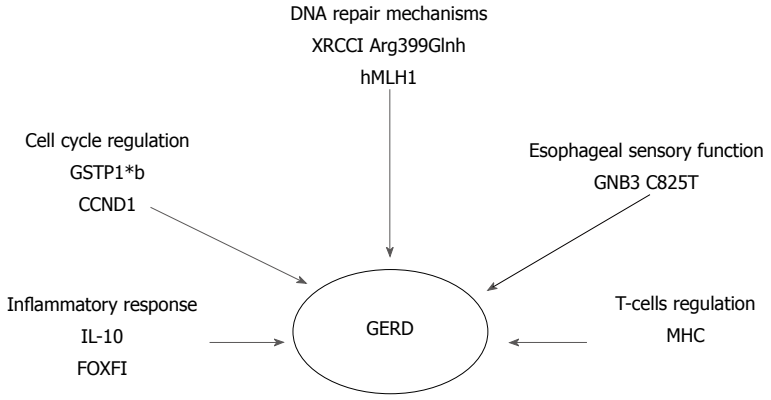

Figure 2 Susceptible risk genes for gastroesophageal reflux disease. Their increased or reduced (DNA repair genes) expression alters different biological pathways. GERD: Gastroesophageal reflux disease; IL-10: Anti-inflammatory cytokine interleukin 10; FOXF1: Forkhead BOX F1; GSTP1*b: Glutathione- S-transferases b allele; CCND1: Cyclin D1 gene; XRCC1: X-ray repair complementing defective repair in Chinese hamster cells 1; Hmlh1: Humal homolog of the *E. coli* DNA mismatch repair gene mutL; GNB3: Guanine nucleotide binding protein beta polypeptide 3; MHC: Major histocompatibility complex.

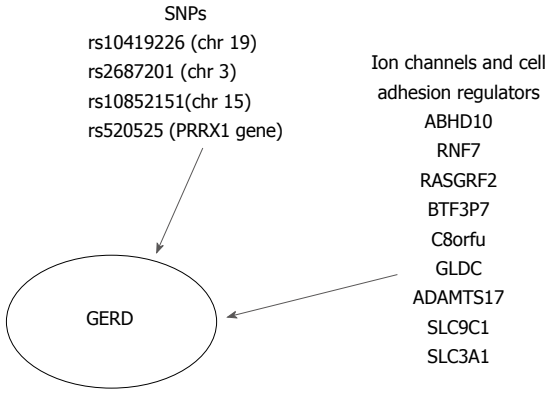

Figure 3 Genetic risk loci associated with the development of gastro-esophageal reflux disease[32,38]. GERD: Gastroesophageal reflux disease; SNPs: Single-nucleotide polymorphisms.

= 0.011). Their effect on gene expression in relevant tissues, such as gastroesophageal junction, esophagus muscularis, esophagus mucosa and stomach, was also evaluated. GERD risk genes influence the regulation of several biological pathways, including the ion channel and the cell adhesion. Moreover, expression trait quantitative loci (eQTL) analyses revealed that these risk genetic loci were enriched for significant eQTLs from GERD-related tissues. The following seven genes, *ABHD10*, *RNF7*, *RASGRF2*, *BTF3P7*, *C8orf4*, *GLDC*, and *ADAMTS17*, located in GERD risk region could be potential GERD risk candidate. Additionally, they pointed at two more ion-channel genes, the SLC9C1 gene (a NA^+/H^+ exchanger) associated with eQTLs in the gastroesophageal junction and the SLC3A1 (an amino-acid transporter) associated with eQTLs in the esophagus mucosa, which is of great interest in terms of treatment with PPIs. Moreover, the authors suggested that the risk genes *ADAMTS17* (rs4965272) and *ADAM10* should be investigated in the future, since *ADAMTS17* participates in numerous biological processes and *ADAM10* controls the e-cadherin proteolytic cleavage in GERD patients. Finally, they attempted to link the identified GERD risk loci with the known therapeutic compounds, by using a computational Connectivity Map analysis. Interesting results were obtained for omeprazole (P = 0.032) and fludroxycortide ($P = 1.04 \times 10^{-4}$). After the Anatomical Therapeutic Chemical index system was taken into account, the class JO4A (anti-tuberculosis drugs) showed high scores after normalization ($P \leqslant 0.033$), as the ion channel genes could be an antituberculosis target. Nevertheless, there was a significant limitation in predicting the total drug regulation effect, despite the fact that it was suggested that drugs affect the expression of these genes (Figure 3)[35].

Genetic factors substantially explain the phenotypic variance of the severity of some GERD symptoms and increase our knowledge of the etiology of the disease. Future genetic studies should define the relation between GERD and its pathophysiological features such as BMI, body fat distribution and hiatal hernia, leading to the identification of biomarkers for GERD prevention and molecular targets for novel treatment. The genetic overlap between GERD, BE and EA may be helpful in future treatments targeting shared molecular pathways involved in pathogenesis of these diseases. Furthermore genetic markers can be discovered to help identify the highest risk individuals for intervention, patients with GERD that will or not progress to EA. That genetic difference could be exploited to determine which patients with GERD are at risk; as such more aggressive screening and treatment could be focused on a clear high risk group[42-44].

CONCLUSION

GERD has proven to be a multivariate disorder, including abnormal anatomical structures and co-morbidities, affected by environmental and genetic factors. The latter has been also found in several studies and in a newly published GWAS, although none of them has

established specific genetic loci with certainty. Further investigation on the mentioned risk genes is needed, in order to evaluate their exact function and role, to probably use them as screening tools or biomarkers and to demonstrate new therapeutic approaches to this increasingly common disease.

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ORIGINAL ARTICLE

Basic Study

Antiviral effects of hepatitis B virus S gene-specific anti-gene locked nucleic acid in transgenic mice

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Abstract

AIM

To assess the antiviral effects of hepatitis B virus (HBV) S gene-specific anti-gene locked nucleic acid (LNA) in transgenic mice.

METHODS

Thirty HBV transgenic mice were acclimatized to laboratory conditions and positive for serum HBV surface antigen (HBsAg) and HBV DNA, were randomly divided into 5 groups (n = 7), including negative control (blank control, unrelated sequence control), positive control (lamivudine, anti-sense-LNA), and anti-gene-LNA experimental group. LNA was injected into transgenic mice by tail vein while lamivudine was administered

by gavage. Serum HBV DNA and HBsAg levels were determined by fluorescence-based PCR and enzyme-linked immune sorbent assay, respectively. HBV S gene expression amounts were assessed by reverse transcription polymerase chain reaction. Positive rates of HBsAg in liver cells were evaluated immunohistochemistry.

RESULTS

Average rate reductions of HBsAg after treatment on the 3rd, 5th, and 7th days were 32.34%, 45.96%, and 59.15%, respectively. The inhibitory effect of anti-gene-LNA on serum HBsAg peaked on day 7, with statistically significant differences compared with pre-treatment (0.96 ± 0.18 *vs* 2.35 ± 0.33, $P < 0.05$) and control values ($P < 0.05$ for all). Average reduction rates of HBV DNA on the 3rd, 5th, and 7th days were 38.55%, 50.95%, and 62.26%, respectively. This inhibitory effect peaked on the 7th day after treatment with anti-gene-LNA, with statistically significant differences compared with pre-treatment (4.17 ± 1.29 *vs* 11.05 ± 1.25, $P < 0.05$) and control values ($P < 0.05$ for all). The mRNA levels of the HBV S gene ($P < 0.05$ for all) and rates of HBsAg positive liver cells ($P < 0.05$ for all) were significantly reduced compared with the control groups. Liver and kidney function, and histology showed no abnormalities.

CONCLUSION

Anti-gene-LNA targeting the S gene of HBV displays strong inhibitory effects on HBV in transgenic mice, providing theoretical and experimental bases for gene therapy in HBV.

Key words: Anti-gene therapy; Hepatitis B virus; Locked nucleic acid; Hepatitis B; Transgenic mice; Anti-sense-therapy

Core tip: We assess the antiviral effects of hepatitis B virus (HBV) S gene-specific anti-gene locked nucleic acid (LNA) in transgenic mice, to provide an experimental basis for gene therapy in patients with Chronic B-related Hepatitis. The inhibitory effect of anti-gene-LNA on serum HBV surface antigen (HBsAg) and HBV DNA peaked on day 7, with statistically significant differences compared with pre-treatment and control values. The mRNA levels of the HBVS gene and rates of HBsAg positive liver cells were significantly reduced compared with the control groups.

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INTRODUCTION

Hepatitis B virus (HBV) is one of the most severe human infectious virus in the world. According to estimates, 240 million individuals globally are chronically infected with HBV[1]. In the past few years the prevalence of chronic HBV infection shows a declining trend throughout the world because of an anti-HBV vaccine and the implementation of successful immunization programs in enzootic zones. Despite improvement in global access to vaccination and treatment, mortality levels remain high[2,3]. Chronic hepatitis B can be effectively and safely treated but a cure remains elusive.

The HBV genome is surrounded by an envelope containing HBV surface antigen (HBsAg) and HBV core antigen (HBcAg) and is a relaxed circular and partially double-stranded DNA[4]. In the nuclei of infected hepatocytes, covalently closed circular DNA (cccDNA) is compounded and sustained at low replication levels are infected with HBV; cccDNA plays a role as the transcription template for all HBV RNAs[5]. Due to cccDNA persistence in a stable form within the hepatocyte nucleus, it remains to a great extent unaffected by current therapies. One of the most important antiviral Drugs includes nucleus(t)ide analogues (NAs) and PEGylated/non-PEGylated interferon-alpha (IFN-α) are widely used to limit viral replication in chronic hepatitis B (CHB) infection[6]. NAs therapy *via* a direct effect on DNA polymerase activity to some extent notably reduces viral load[7]. Unfortunately, due to somewhat poor response and/or drug resistance, these therapies without achieve a therapeutic effect[8]. Therefore, the development of a novel therapeutic strategy to repress HBV replication is of great significance in saving lives of CHB patients.

Recently, anti-sense-LNA was shown to effectively inhibit HBV replication and expression *in vitro* models[9]. However, anti-sense therapy cannot cut off the replication and transcription of viral genes from the source. It can only interfere with the synthesis of viral protein at the level of expression, and is prone to stop drug rebound. In anti-gene therapy, triplex-forming oligonucleotides (TFOs) provides a promising approach to bind in the major groove of duplex DNA at polypurine or polypyrimidine stretches in a sequence-specific manner[10]. TFOs prevent the association of target DNA, polymerase, and transcription factors, down-regulating target genes[11-13]. A recent and promising technological progress is the development of locked nucleic acid (LNA), which not only enhances binding to its target sequence while being resistant to nuclease degradation, but also shows minimal toxicity[14-16]. Compared with anti-sense therapy, it has the advantage of blocking virus gene replication and transcription from the source.

The aim of this study was to design liposome based transport of a LNA modified oligonucleotide to inhibit HBV DNA express in transgenic mice, the final objective was to assess the antiviral effects of HBV S gene-specific anti-gene LNA in transgenic mice.

MATERIALS AND METHODS

Anti-gene-LNA synthesis and modification

According to the HBV S encoding chain, anti-gene-LNA was designed using the Walk function of the RNA structure software to select ΔG37 value of small fragments; by Blast analysis and homology sequence features, the synthesis and modification of anti-gene-LNA (5′-TaccTcTtgtA-3′; uppercase and lower case letters represent LNA and DNA, respectively) were performed by Shanghai biotechnology limited company.

Animals

HBV transgenic mice (n = 35; 18 males and 17 females) weighing 19-23 g were purchased from the Guangzhou Military Air Force Hospital of the People's Liberation Army of China. All mice were positive for serum HBsAg and HBV DNA. They were bred and housed in pathogen-free conditions at 25 ℃ ± 2 ℃ under a 12 h:12 h light-dark cycle, with food and water provided ad libitum unless otherwise specified. All animal care and experimental procedures were approved by the Institutional Ethnics Committee of Youjiang Medical College for Nationalities.

Mice were randomly divided into five groups (n = 7 each), including negative control (blank control, unrelated sequence control), positive control (lamivudine, anti-sense-LNA), and anti-gene-LNA experimental group. The lamivudine control group was administered daily gavage of 2 mg/kg for 7 d; the remaining groups were injected (400 μL 5% glucose-liposome containing the corresponding drug) *via* the tail vein at 1, 3, and 5 d, respectively. Blood samples were collected from the orbital vein before and after injection on 3rd, 5th and 7th days, respectively, and centrifuged at 5000 r/min for 5min. The resulting serum was stored at -20 ℃ until use. On the 7th day, all mice were sacrificed by cervical dislocation under anesthesia. Liver and kidney samples were obtained for histological assessments, ultrastructural examinations, and immunohistochemistry.

Measurement of serum HBsAg levels by enzyme-linked immune sorbent assay

Serum HBsAg levels were quantified with an enzyme-linked immune sorbent assay enzyme-linked immune sorbent assay (ELISA) kit according to the manufacturer's protocol. ELISA kits were purchased from Lizhu Biology Company, Zhuhai, China.

Measurement of HBV DNA levels by Fluorescence based PCR

Serum HBV DNA levels were quantified with a diagnostic kit for the quantification of HBV DNA, according to the manufacturer's protocol. HBV DNA diagnostic kits were purchased from Daan Gene Company, Guangzhou, China. Briefly, 30 μL serum was added to 70 μL DNA extract and oscillated for 15 s, incubated at 100 ℃ for 10 min, and centrifuged at 12000 r/min for 5 min. Then, DNA samples (20 μL) were added to 30 μL of PCR reaction mix and centrifuged at 8000 r/min for 5 s. PCR was performed as follows: 93 ℃for 2 min; 10 cycles of 93 ℃ for 45 s and 55 ℃for 1 min; 30 cycles of 93 ℃for 30 s and 55 ℃ for 45 s; 40 ℃for 20 s. DNA levels were determined based on a standard curve for HBV.

Measurement of HBV S gene expression levels in the liver by reverse transcription polymerase chain reaction

Total RNA was extracted from liver samples with TRNzol Universal Reagent kit (TIANGEN), and concentrations were determined by spectrophotometry. Reverse transcription was performed with Fast Quant RT Kit (TIANGEN), according to the manufacturer's instructions. Briefly, RT was carried out in a final volume of 20 μL containing 5 × g DNA buffer (2 μL), Total RNA (1 μL), RNase-free ddH_2O (7 μL) (incubated at 42 ℃ for 3 min), 10 × Fast RT buffer (2 μL), RT Enzyme Mix (1 μL), FQ-RT Primer Mix (2 μL), and RNase-Free ddH_2O (5 μL), total 20 μL. The mixture was incubated at 42 ℃ for 15 min and 95 ℃ for 3 min. PCR amplification of the S gene was performed with the following primers: Forward, 5′-CTGCCTCTCCCTTATCGTCA-3′; Reverse, primer5′-TGGCAAGGACCCATAACTTC-3′.

The following temperature protocol was used: 94 ℃ for 3 min; 30 cycles of 94 ℃ for 30 s, 55 ℃ for 30 s, and 72 ℃ for 1 min; 72 ℃ for 5 min. The amplicon was 830 bp. The glyceraldehyde phosphate dehydrogenase (GAPDH; reference gene) band in electrophoresis was used to determine the relative HBV S gene expression levels.

Immunohistochemistry

To detect HBsAg in liver cells, the sections were dewaxed in xylene and rehydrated in graded alcohol. The endogenous peroxidase activity was suppressed by 3% hydrogen peroxide for 15 min. After rinsing twice in phosphate-buffered saline (PBS), antigen retrieval was performed by immersing the sections in 10 mmol/L sodium citrate buffer (pH 6.0) and heated for 15 min in a microwave oven. The sections were then treated for 4 ℃ for 18 h with mouse anti-HBsAg monoclonal antibody (1:100). PBS was used as a negative control. After three washes with PBS, the sections were subsequently treated with biotinylated goat anti-rabbit immunoglobulin for 15 min and horseradish peroxidase-streptavidin complex for 15 min. The slides were then washed three times with PBS and incubated in DAB for 5 min and counterstained with hematoxylin for 30 s. After dehydration with graded alcohol, the slides were mounted and analyzed under an Olympus BX53 inverted microscope (Olympus, Japan).

Histology

To assess the morphological changes of the liver and kidney, formalin-treated tissue samples were paraffin embedded. Serial sections with 4 mm thickness were obtained and stained with hematoxylin and eosin (H

Table 1 Serum hepatitis B surface antigen levels in transgenic mice (n = 7, mean ± SD)

Groups	Before treatment	After treatment			
		Day 1	Day 3	Day 5	Day 7
Blank	2.34 ± 0.22	2.33 ± 0.23	2.32 ± 0.31	2.37 ± 0.30	2.35 ± 0.21
USQ	2.35 ± 0.36	2.34 ± 0.25	2.37 ± 0.31	2.31 ± 0.45	2.36 ± 0.38
LAM	2.33 ± 0.28	2.31 ± 0.38	2.27 ± 0.24	2.21 ± 0.23	2.15 ± 0.19
Anti-S-LNA	2.31 ± 0.27	2.03 ± 0.28	1.61 ± 0.11	1.55 ± 0.16	1.33 ± 0.26
Anti-G-LNA	2.35 ± 0.33	1.92 ± 0.40	1.59 ± 0.32[a,b]	1.27 ± 0.29[a,b]	0.96 ± 0.18[a,b,c]

[a]Indicates significant differences between pre-treatment *vs* Anti-G-LNA (P < 0.05); [b]Indicates significant differences between Blank, USQ, LAM group *vs* Anti-G-LNA (P < 0.05); [c]Indicates significant differences between Anti-S-LNA *vs* Anti-G-LNA (P < 0.05). Values are mean ± SD. Blank: Blank control group, 5% glucose-liposome administered by tail vein injection; USQ: Unrelated sequence; LAM: Lamivudine; Anti-S-LNA: Anti-sense-LNA; Anti-G-LNA: Anti-gene-LNA; LNA: Locked nucleic acid.

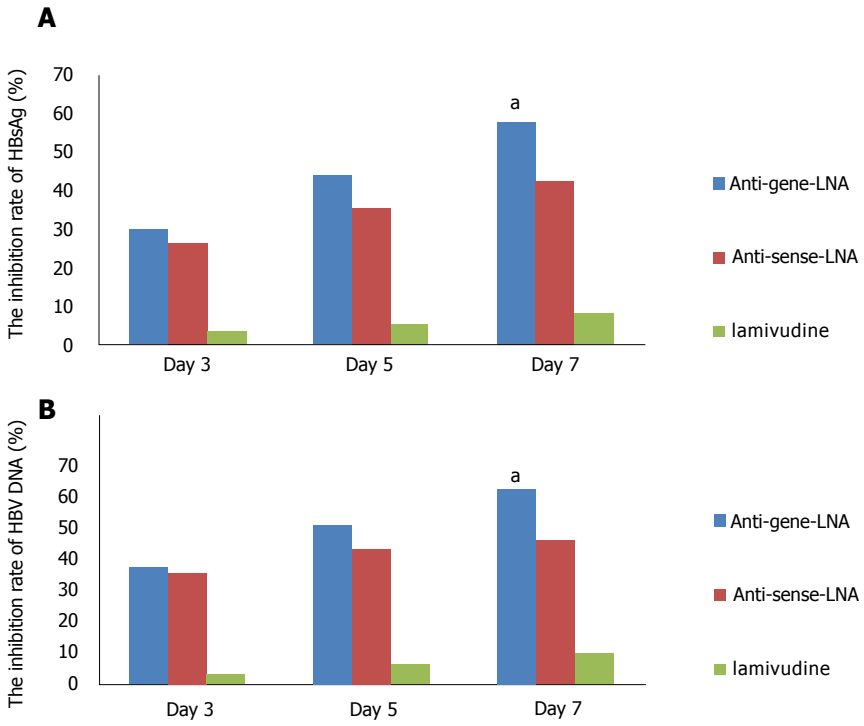

Figure 1 Inhibition of hepatitis B virus deoxyribonucleic acid and hepatitis B surface antigen by anti-gene-locked nucleic acid, anti-sense-locked nucleic acid, and lamivudine. A: The inhibition rate of HBsAg; B: The inhibition rate of hepatitis B virus (HBV) deoxyribonucleic acid. [a]Significant (P < 0.05) HBsAg and HBV DNA expression alterations between lamivudine, anti-sense-LNA *vs* anti-gene-LNA. Values are mean ± SD. LNA: Locked nucleic acid; HBsAg: HBV surface antigen.

and E). Observation was performed under an Olympus BX53 inverted microscope (Olympus, Japan).

Statistical analysis

Quantitative data are mean ± SD, and were analyzed with the SPSS 13.0 software. Groups were compared by one-way analysis of variance (ANOVA) followed by the Tukey's multiple range post hoc test. P < 0.05 was considered statistically significant.

RESULTS

Serum HBsAg levels

The inhibitory effect on serum HBsAg was assessed by ELISA on the 3rd, 5th, and 7th days, respectively, after treatment. Average rate reductions after treatment on the 3rd, 5th, and 7th days were 32.34%, 45.96%, and 59.15%, respectively. The inhibitory effect of anti-gene-LNA on serum HBsAg peaked on day 7, with statistically significant differences compared with pre-treatment and control values (P < 0.05). This suggests that anti-gene-LNA significantly inhibited HBsAg in mice in a time-dependent manner (Table 1 and Figure 1A).

HBV DNA levels

The inhibitory effect of anti-gene-LNA on HBV DNA was analyzed by fluorescence based PCR on the 3rd, 5th, and 7th days after treatment, respectively. Average reduction rates on the 3rd, 5th, and 7th days were 38.55%, 50.95%, and 62.26%, respectively. This inhibitory effect peaked on the 7th day after treatment with anti-gene-LNA, with statistically significant differences compared with pre-

Table 2 Effects of anti-gene-locked nucleic acid on hepatitis B virus deoxyribonucleic acid replication and expression in transgenic mice (n = 7, mean ± SD; × 10^3 IU/mL)

Groups	Before treatment	After treatment			
		Day 1	Day 3	Day 5	Day 7
Blank	10.81 ± 1.15	10.80 ± 0.78	10.86 ± 1.85	10.80 ± 1.19	10.77 ± 1.25
USQ	11.12 ± 0.87	11.25 ± 0.94	11.16 ± 0.96	11.06 ± 0.85	11.08 ± 0.89
LAM	10.96 ± 1.08	10.93 ± 1.12	10.62 ± 0.89	10.07 ± 1.37	9.73 ± 1.17
Anti-S-LNA	10.92 ± 1.09	8.94 ± 0.89	6.91 ± 1.26	5.48 ± 0.97	5.79 ± 0.92
Anti-G-LNA	11.05 ± 1.25	9.12 ± 0.96	6.79 ± 1.16[a,b]	5.42 ± 1.12[a,b]	4.17 ± 1.29[a,b,c]

[a]Indicates significant differences between pre-treatment *vs* Anti-G-LNA (P < 0.05); [b]Indicates significant differences between Blank, USQ, LAM group *vs* Anti-G-LNA (P < 0.05); [c]Indicates significant differences between Anti-S-LNA *vs* Anti-G-LNA (P < 0.05). Values are mean ± SD. Blank: Blank control group, 5% glucose-liposome administered by tail vein injection; USQ: Unrelated sequence; LAM: Lamivudine; Anti-S-LNA: Anti-sense-LNA; Anti-G-LNA: Anti-gene-LNA; LNA: Locked nucleic acid.

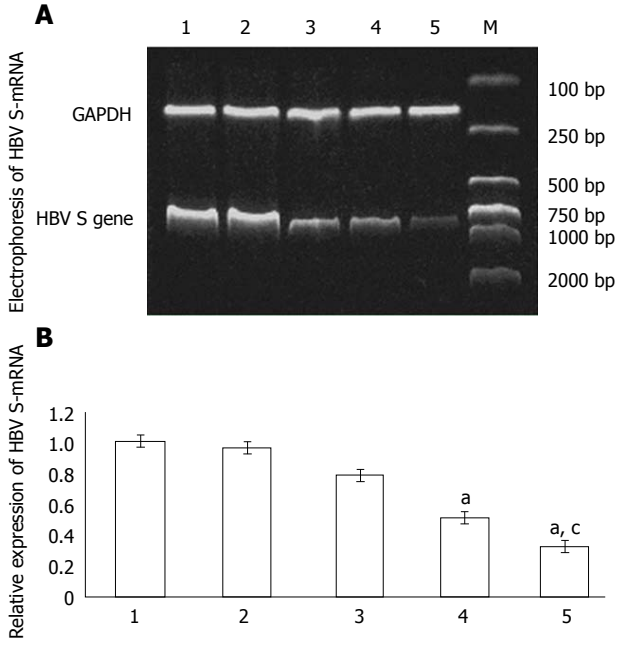

Figure 2 Hepatitis B virus S-mRNA expression levels in the liver of transgenic mice (mean ± SD, n = 7). A: Electrophoregram showing HBV S and GAPDH bands; B: Relative expression levels of HBV S-mRNA. [a]Significant (P < 0.05) S-mRNA expression alterations between blank (1), unrelated sequence (2), lamivudine (3) *vs* Anti-sense-LNA (4) and Anti-gene-LNA (5); [c]Significant (P < 0.05) S-mRNA expression alterations between Anti-sense-LNA (4) *vs* Anti-gene-LNA (5). Values are mean ± SD. M: DNA marker; LNA: Locked nucleic acid; HBV: Hepatitis B virus

treatment and control values (P < 0.05). This suggests that anti-gene-LNA significantly inhibits HBV DAN in mice in a time-dependent manner (Table 2 and Figure 1B)

HBV S gene expression levels in the liver

The average grayscale values of lanes 1-5 for HBV S-DNA (with GAPDH set to 1) were 1, 0.96, 0.78, 0.51, and 0.32, respectively. Quantitation of HBV S gene expression levels in the liver after treatment with anti-gene-LNA revealed a significant decrease compared with pre-treatment (P < 0.05) or control (P < 0.05) values (Figure 2).

HBsAg positive cells in liver tissues

Figure 3 shows representative immunohistochemical staining data for HBsAg positive cells in liver tissues. No significant differences were observed in the rates of HBsAg positive liver cells among the blank control, unrelated sequence control and lamivudine control groups. Meanwhile, 47% of liver cells were positive for HBsAg after treatment with anti-sense-LNA verify, a rate significantly lower than those of the blank control, unrelated sequence control, and lamivudine control groups (P < 0.05); the positive expression rate of HBsAg in liver cells in the anti-gene-LNA treatment group was 31%, significantly lower than those of all control groups (P < 0.05)

Histological observations

H&E staining was used to assess the effects of anti-gene-LNA and liposomes on the histological features of the liver and kidney. Liver and kidney sections stained with H and E showed no significant differences between the anti-gene-LNA group and controls, suggesting that anti-gene-LNA had no obvious toxicity on liver and kidney at the histological level (Figures 4 and 5).

DISCUSSION

The present study assessed serum HBsAg, HBV DNA, and HBV S gene expression levels in transgenic mice. The results demonstrated that HBV amounts were significantly reduced after injection of anti-gene- LNA on 7th day, shows a significant difference compared with before treatment and control groups (P < 0.05). Significantly less positive HBsAg cells in liver tissues were obtained after treatment with anti-gene-LNA compared with the control groups, indicating that anti-gene-LNA transacted by cationic liposomes can effectively enter nucleolus in the liver of transgenic mice after tail vein injection and play a role in reducing HBV DNA replication and transcription.

We successfully used anti-sense-LNA to inhibit the translation and expression of HBV mRNA in vitro and in vivo models in previous study[15], due to the basic principle of anti-sense-LNA targeting specific mRNAs by annealing complementary oligonucleotides[17-20]. In the simplest form, anti-sense-LNAs are introduced into the liver cell to down-regulate gene expression

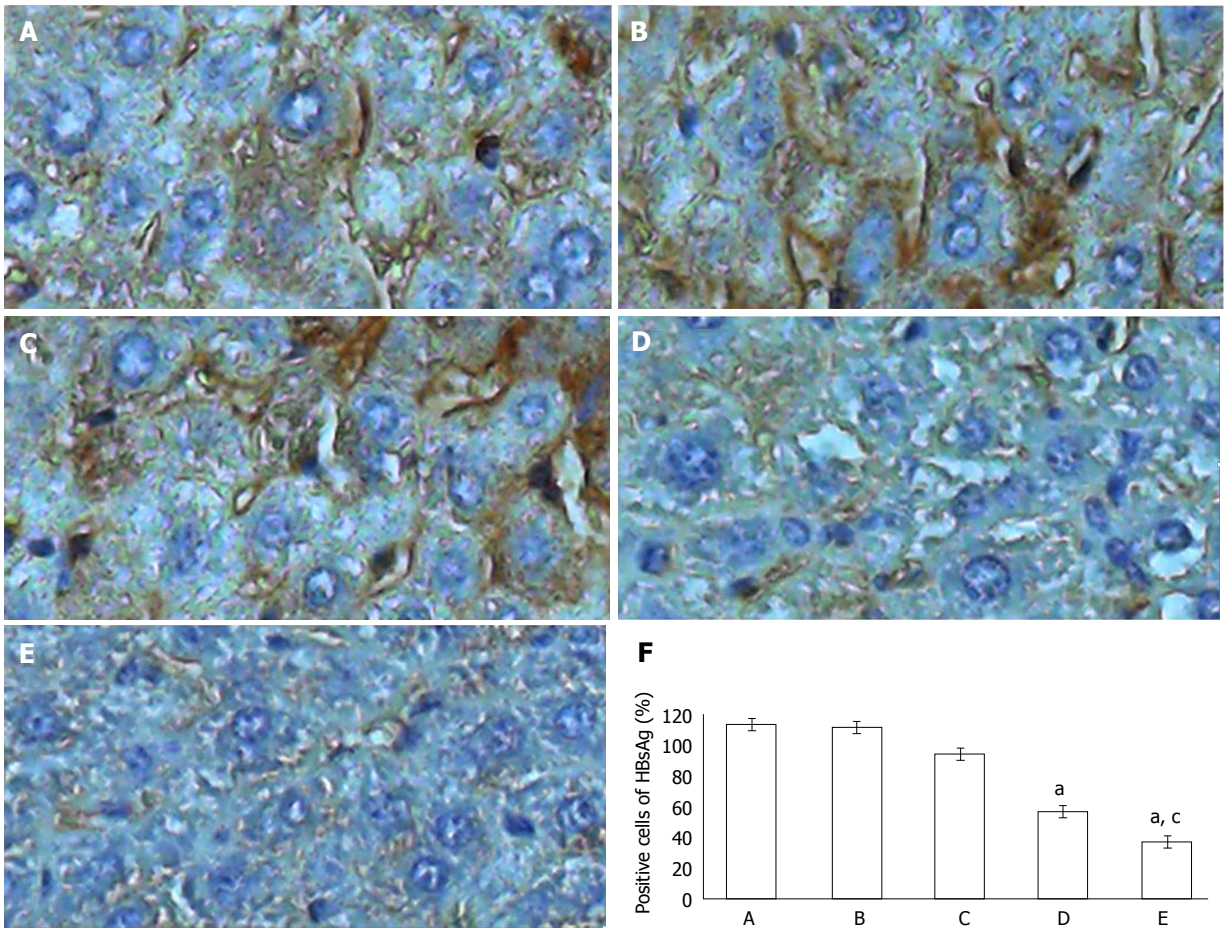

Figure 3 Immunohistochemical detection (× 200) of hepatitis B surface antigen positive cells in liver tissues of transgenic mice. [a]Significant ($P < 0.05$) HBsAg positive cells expression alterations between blank (A),unrelated sequence (B), lamivudine (C) *vs* anti-sense-LNA (D) and anti-gene-LNA (E); [c]Significant ($P < 0.05$) HBsAg positive cells expression alterations between anti-sense-LNA (D) *vs* anti-gene-LNA (E). Values are mean ± SD; F: Positive HBsAg cells. LNA: Locked nucleic acid; HBV: Hepatitis B virus; HBsAg: HBV surface antigen.

by interfering with the translation of mRNA instead of DNA transcription, *i.e.*, they are involved in posttranscriptional rather than transcriptional gene regulation. Therefore, treatment with anti-sense-LNA does not achieve satisfactory effects. TFOs have emerged as potential regulators of biological activity for direct modifications of genomic DNA at selected sites through mutagenesis or homologous recombination and changing the anti-gene therapeutic method[21-23]. In the present study, anti-gene-LNA was assessed for its antiviral effects in transgenic mice. The results showed that the inhibitory effect of anti-gene-LNA peaked on the 7th day, with a statistically significant difference compared with the anti-sense-LNA group ($P < 0.05$). However, compared with other nucleic acid-based approaches, TFOs faces challenges such as oligonucleotides (ONs) targeting ability and the stability of the TFOs in a genomic context. In order to efficient, ON should increase target DNA selectively, easily, stability, and with high affinity. Until now, due to the low of affinity of TFOs or their DNA targets, the method of anti-gene-DNA hampered to down-regulate and control the expression level of genomic DNA. The anti-gene-LNA sugar unit not only significantly enhances triplex stability but also partly relieves sequence restriction constraints[24-26]. The present study provided evidence that once in the nucleus, an integrated HBV DNA sequence composed of LNA and DNA oligonucleotides (anti-gene-LNA) effectively targeted HBV, therefore inhibiting its replication at the transcription level.

HBV DNA is easily mutated compared to other DNA viruses during replication; one of the important reasons is that HBV DNA polymerase lacks a proofreading function[27], which can cause HBV mutations to occur at a 10-fold higher frequency[28]. This results in decreased susceptibility or increased resistance in anti-HBV treatment. HBV contains 4 overlapping open reading frames (ORFs) encoding the polymerase (P), core (C), surface antigen (S), and X proteins[29]. The S gene region is one of the most important open reading frames of the HBV genome, encoding a protein forming an important part of HbsAg[30,31]. Thus, the S gene is not only closely associated with virus replication, transcription, assembly, and secretion processes, but also with cellular and humoral immune responses induced by the virus.

Taken together, this study showed an effective

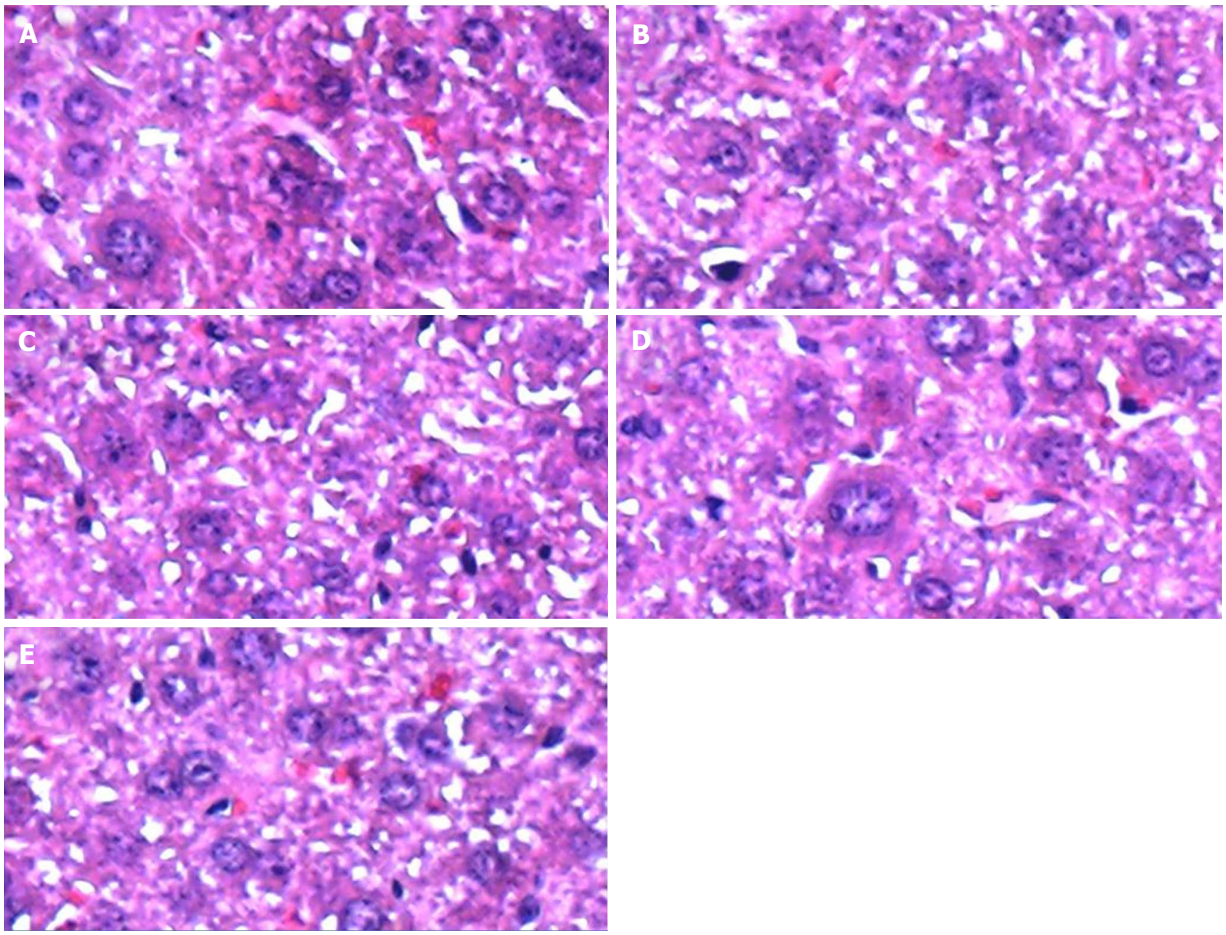

Figure 4 Morphological changes in liver sections obtained from transgenic mice infected with hepatitis B virus. Mice were sacrificed, and the livers were harvested and prepared for observation on the 7th d after treatment. A: Blank control group; B: Unrelated sequence control group; C: Lamivudine control group; D: Anti-sense-LNA treatment control group; E: Anti-gene-LNA treatment group. Liver sections were stained with hematoxylin and eosin (original magnification, × 200). There were no significant morphological changes in the liver among groups. LNA: Locked nucleic acid.

strategy that with liposome based transport of a LNA modified oligonucleotide to inhibit HBV DNA expression in transgenic mice. The new treatment strategy of repressing HBV DNA replication is usefulness and worth further studying. Based on the data presented herein highlight the usefulness of anti-gene-LNA mediated silencing HBV DNA replication and transcription bring forth innovative ideas and potentially viable tool for gene therapy.

ARTICLE HIGHLIGHTS

Research background

Hepatitis B virus (HBV) is one of the most severe human pathogens. It is reported that 240 million individuals globally are chronically infected with HBV and current antivirals cannot clear the infection or adequately suppress disease.

Research motivation

Despite improvement in global access to vaccination and treatment, mortality levels remain high. Chronic hepatitis B can be effectively and safely treated but a cure remains elusive.

Research objectives

The aim of this study is to inhibit HBV DNA expression with anti-gene locked nucleic acid (LNA) in transgenic mice.

Research methods

The aim of this study was to design liposome based transport of a LNA modified oligonucleotide to inhibit HBV DNA express in transgenic mice, the final objective was to assess the antiviral effects of HBV S gene-specific anti-gene LNA in transgenic mice.

Research results

Average rate reductions of HBsAg, HBV DNA, mRNA levels of the HBV S gene and the rate of HBsAg positive liver cells, with statistically significant differences compared with pre-treatment and control values ($P < 0.05$ for all). Liver and kidney function, and histology showed no abnormalities.

Research conclusions

Anti-gene-LNA transacted by cationic liposomes can effectively enter nucleolus in the liver of transgenic mice after tail vein injection and play a role in reducing HBV DNA replication and transcription.

Research perspectives

Based on the data presented herein highlight the usefulness of anti-gene-LNA

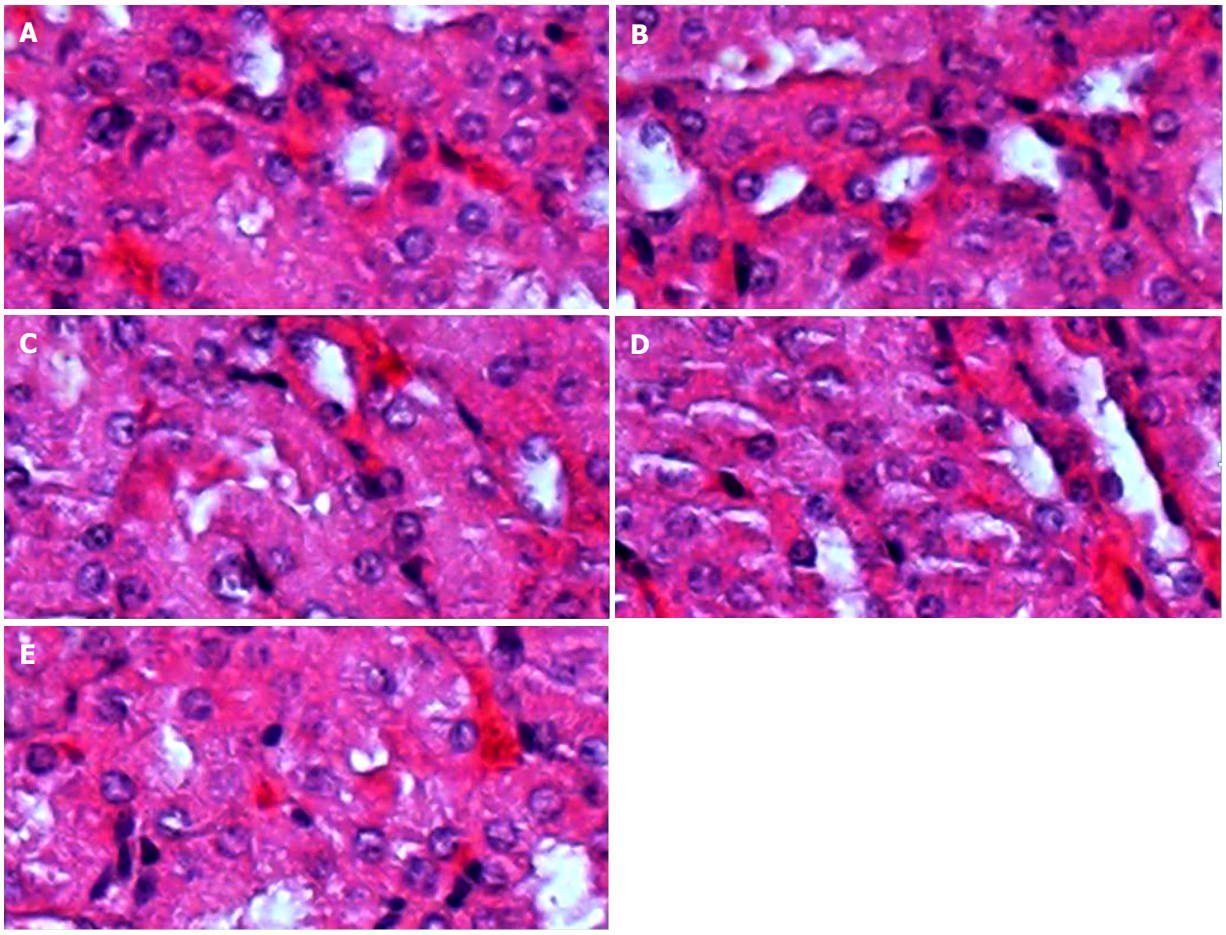

Figure 5 Morphological changes in kidney sections obtained from transgenic mice infected with hepatitis B virus. Mice were sacrificed, and the kidneys were harvested and prepared for observation on the 7th d after treatment. A: Blank control group; B: Unrelated sequence control group; C: Lamivudine control group; D: Anti-sense-LNA treatment control group; E: Anti-gene-LNA treatment group. Kidney sections were stained with hematoxylin and eosin (original magnification, × 200). There were no significant morphological changes in the kidney among groups. LNA: Locked nucleic acid.

mediated silencing HBV DNA replication and transcription bring forth innovative ideas and potentially viable tool for gene therapy.

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ORIGINAL ARTICLE

Case Control Study

Negative impact of hepatitis B surface seroclearance on prognosis of hepatitis B-related primary liver cancer

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Author contributions: Lou C and Du Z designed research; Lou C, Du Z, Bai T and Bi LW treated patients and collected material and clinical data; Gao YT collected and sorted material and clinical data; Lou C and Bi LW performed the assays and analyzed data; Lou C wrote the paper.

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Abstract

AIM

To assess the impact of hepatitis B surface (HBsAg) seroclearance on survival outcomes in hepatitis B-related primary liver cancer.

METHODS

Information from patients with hepatitis B-related liver cancer admitted in our hospital from 2008-2017 was retrieved. Cases diagnosed with HBsAg (-) and HBcAb (+) liver cancer were included in the HBsAg seroclearance (SC) group. HBsAg (+) liver cancer patients strictly matched for liver cancer stage (AJCC staging system, 8th edition), Child-Pugh score, and first diagnosis/treatment method (surgery, ablation and TACE) were assigned to the HBsAg non-seroclearance (NSC) group. Then, clinical, pathological and survival data in both groups were assessed.

RESULTS

The SC and NSC groups comprised of 72 and 216 patients, respectively. Patient age (P < 0.001) and platelet count (P = 0.001) in the SC group were significantly higher than those of the NSC group. SC group patients who underwent surgery had more intrahepatic cholangiocarcinoma (ICC) and combined HCC-CC (CHC) cases than the NSC group, but no significant differences in tumor cell differentiation and history of liver cirrhosis were found between the two groups. The numbers of interventional treatments were similar in both groups (4.57 *vs* 5.07, P > 0.05). Overall survival was lower in the SC group than the NSC group (P = 0.019), with 1-, 3-, and 5-year survival rates of 82.1% *vs* 85.1%, 43.2% *vs* 56.8%, and 27.0% *vs* 45.2%, respectively. Survival of patients with AJCC stage Ⅰ disease in the SC group was lower than that of the NSC group (P = 0.029).

CONCLUSION

Seroclearance in patients with hepatitis B-related primary liver cancer has protective effects with respect to tumorigenesis, cirrhosis, and portal hypertension but confers worse prognosis, which may be due to the frequent occurrence of highly malignant ICC and CHC.

Key words: Primary liver cancer; Hepatitis B surface; Hepatitis B surface seroclearance; Prognosis; Chronic hepatitis B

Core tip: Through strict case-control, we eliminated prognostic confounding factors, such as tumor stage, Child-Pugh score, and therapeutic mode, to determine the impact of hepatitis B surface (HBsAg) seroclearance (SC) on the prognosis of HBV related liver cancer,. Statistical analysis shows that although HBsAg SC is protective in tumorigenesis, liver cirrhosis, and portal hypertension, the prognosis of HBsAg SC patients with primary liver cancer is worse than that of controls.

Lou C, Bai T, Bi LW, Gao YT, Du Z. Negative impact of hepatitis B surface seroclearance on prognosis of hepatitis B-related primary liver cancer. *World J Clin Cases* 2018; 6(8): 192-199 Available from: URL: http://www.wjgnet.com/2307-8960/full/v6/i8/192.htm DOI: http://dx.doi.org/10.12998/wjcc.v6.i8.192

INTRODUCTION

Chronic infection with hepatitis B virus (HBV) is the most common cause of liver cancer in Asia, especially in China. In Asia, about 60% of liver cancer cases are associated with HBV infection[1]. Hepatitis B surface antigen (HBsAg) seroclearance is considered the gold standard for hepatitis B viral clearance and chronic hepatitis B cure, and is now regarded as the end point of antiviral therapy[2,3]. Approximately 0.1%-0.8% of adult patients with chronic HBV infection achieve HBsAg seroclearance in the course of natural development[4,5]. Antiviral therapy could also lead to HBsAg seroclearance in some patients, improving the clinical outcome of individuals with chronic hepatitis B infection[6,7].

After HBsAg seroclearance in these patients, the liver retains very low HBVDNA levels. Compared with chronic hepatitis B patients with positive HBsAg, a further decrease of HBVDNA *in vivo* results in significantly improved liver histology and biochemistry. However, the incidence of liver cancer remains in patients with seroclearance[5,6,8,9]. A history of cirrhosis and age above 50 years at HBsAg seroclearance are high risk factors for liver cancer[10,11]. Existing studies have confirmed that HBV seroclearance could effectively improve the prognosis of liver cancer patients with positive HBsAg[12,13]. However, whether HBsAg seroclearance, which indicates a further decrease in viral load, affects the prognosis of patients with liver cancer remains unclear. Because of the small number of such cases, it is currently difficult to have an effective patient control group, leading to few studies in this field. Moreover, inconclusive findings have been reported by the small amount of studies available[14].

In this study, the clinicopathological characteristics of liver cancer patients with HBsAg seroclearance were assessed with strict case control and elimination of confounding factors. The correlation between HBsAg seroclearance and the prognosis of patients with liver cancer was established.

MATERIALS AND METHODS

Patients with primary liver cancer admitted to our hospital from 2008 to 2017 were included. Hepatitis markers, auto-antibodies in liver disease, drinking history, and other etiological data were recorded. Patients with underlying liver diseases (hepatitis C virus, autoimmune liver disease, alcoholic liver disease, cryptogenic cirrhosis and hepatolithiasis) were excluded. Serum HBV DNA levels are routinely detected in HCC patients with HBsAg (-) and HBcAb (+) except for occult hepatitis B (OHB). Then liver cancer patients characterized by HBsAg (-) and HBcAb (+) were selected as the HBsAg seroclearance (SC) group. Those with HBsAg (+) constituted the HBsAg non-seroclearance (NSC) group. According to the consistent principles of the Child-Pugh scoring system for liver function and the 8th edition of the AJCC/UICC staging system for liver cancer and treatment methods, patients in the SC group were strictly matched with those of the NSC group at a proportion of 1:3. According to Child-Pugh scores, the patients were divided into three grades, including A, B, and C. Based on the initial treatment after diagnosis, the patients were divided into surgical resection, ablation, and TACE groups. According to the 8th edition of the AJCC/UICC staging system for liver cancer, the patients were divided into stages Ⅰ, Ⅱ, Ⅲ, and Ⅳ. To accurately reflect treatment responses and the prognosis of patients with liver cancer, AJCC stage

Ⅳ cases were excluded. Meanwhile, in the TACE group, only the patients who received at least two intervention treatments were selected, which fully reflected TACE efficacy.

The hepatitis B status in all patients was determined at diagnosis. HBsAg status was re-examined after the initial treatment and during follow-up. Serum HBsAg was tested quantitatively [Architect assay, Abbott Laboratories, Chicago, Illinois, United States; lower limit of detection (LLOD), 0.05 IU/mL]. Serum HBV DNA levels were measured by using a real-time PCR assay (Roche Laboratories; Basel, Switzerland; LLOD, 100 IU/mL). Before the initial treatment, routine examinations, including routine blood tests, liver function, coagulation function tests, serum-alpha fetoprotein assessment, abdominal ultrasound, contrast ultrasound, enhanced CT, and/or enhanced MRI were performed to determine the diagnosis of liver cancer, tumor size and number, the status of macrovascular invasion, and distant metastasis, as well as liver cancer stage (8^{th} edition of the AJCC/UICC staging system for liver cancer). Patients treated by surgery were routinely examined by ICG. According to liver cancer stage, Child-Pugh score, liver volume obtained from CT scan, ICG findings, and patient willingness, the subjects were respectively selected to undergo surgical excision, ablation therapy, or TACE after the initial diagnosis. Tumor pathology, cell differentiation, underlying liver disease, and other parameters were recorded after surgery. The numbers of interventional treatments in the TACE group were recorded.

All patients were followed up after treatment, and overall survival (OS) was the only main evaluation index. The initial diagnostic time of liver cancer was used as the starting time. The deadline for follow up was December 31, 2017, and survival time was recorded.

Statistical analysis

Continuous data were expressed as mean ± standard deviation (SD). Tumor size and follow-up time were represented by median. *T*-test was used for comparison. Count data were expressed as frequency and proportion, and assessed by the χ^2 test. The Kaplan-Meier method was used for survival analysis, and the log-rank test for comparison.

RESULTS

The clinical data of liver cancer patients in a single center for ten years were retrieved. Of the 4745 patients with liver cancer, there were 1772 cases of hepatitis B-related liver cancer. Among them, 91 cases were diagnosed as liver cancer patients with HBsAg seroclearance, accounting for 5.14% (91/1772), of which five patients were excluded from the analysis due to incomplete data or loss to follow-up. Considering the short life span of patients with advanced liver cancer and poor therapeutic responses, six AJCC stage Ⅳ liver cancer patients were excluded, and eight additional patients were excluded for only receiving a single TACE treatment, which was not suitable for evaluating treatment efficacy. Finally, the clinical data of 72 patients with liver cancer in the SC group were collected. The serum HBVDNA levels of patients in the SC group were all negative and OHC were excluded. Meanwhile, 216 matched patients in the NSC group were enrolled according to a proportion of 1:3.

The baseline characteristics of these patients are shown in Table 1. Median patient age in the SC group was significantly higher than that of the NSC group (63.5 ± 8.9 years *vs* 57.0 ± 9.0 years, $P < 0.001$). Due to Child-Pugh score matching, there were no significant differences in coagulation, albumin, and bilirubin between the two groups, but platelet levels in the SC group were significantly higher than those of the NSC group (163.2 ± 87.5 *vs* 126.7 ± 76.2, $P = 0.001$).

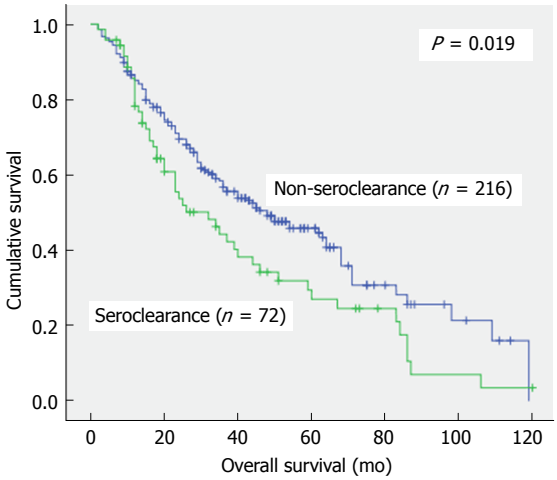
Stratification was carried out according to the treatment method, with 26 patients and 78 patients treated by surgery in the SC and NSC groups, respectively. The clinical data of the above patients are shown in Table 2. Consistent with the overall data, patient age and platelet levels in the SC group were significantly higher than those of the NSC group. A significant difference was found in pathological types of liver cancer between the two groups ($P = 0.002$). The SC group had more ICC and CHC cases compared to the NSC group, but there were no significant differences in tumor cell differentiation and history of cirrhosis between the two groups. A total of 23 patients in the SC group were administered interventional therapy, versus 69 patients in the NSC group. Based on this stratification, patients in the SC group also showed differences in age and platelet levels, which was consistent with the overall data. There was no statistical difference in the number of interventional treatments between the two groups (4.57 *vs* 5.07, $P > 0.05$), indicating the consistency of intervention intensity.

All patients in the SC and NSC groups were followed up, with median follow-up times of 20 mo and 33 mo, respectively, indicating no significant difference between the two groups ($P > 0.05$). As shown in Figure 1, overall survival in the SC group was lower than that of the NSC group ($P = 0.019$). The 1-, 3- and 5-year survival rates were 82.1%, 43.2% and 27.0% in the SC group, respectively, which were significantly lower than those of the NSC group (85.1%, 56.8%, and 45.2%, respectively). Stratification analysis was further carried out according to tumor stage (Figure 2); overall survival in patients with AJCC stage I disease in the SC group was significantly lower than that of the NSC group ($P = 0.029$). Meanwhile, the 1-, 3- and 5-year survival rates were 84.6%, 53.6% and 33.8% in the SC group, respectively, which were significantly lower than those of the NSC group (93.7%, 79.3%, and 66.1%, respectively). Although AJCC stage Ⅱ and Ⅲ patients in the SC group also showed trends of lower survival rates, statistical significance was not achieved.

Table 1 Baseline clinicopathological characteristics

Variables	Seroclearance (n = 72)	Non-seroclearance (n = 216)	P value
Ages [median (range)]	63.5 ± 8.9	57.0 ± 9.0	< 0.001
Sex (male:female)	59:17	171:45	0.871
Hemoglobin (mg/dL)	130.6 ± 28.0	135.3 ± 21.7	0.144
Platelet count (× 10^9/μL)	163.2 ± 87.5	126.7 ± 76.2	0.001
PTA (%)	90.5 ± 20.7	87.7 ± 18.5	0.282
Albumin (g/L)	40.5 ± 5.6	40.1 ± 5.9	0.675
AST (IU/L)	33.3 ± 28.6	39.9 ± 36.7	0.163
ALT (IU/L)	36.9 ± 40.7	42.4 ± 41.7	0.327
TBil (μmol/L)	21.2 ± 20.9	19.8 ± 17.5	0.567
AFP (ng/mL)			
⩽ 15	36	100	0.456
15-200	19	54	
⩾ 200	17	62	
Size of tumor (cm) [median (range)]	5.4 (1-15.4)	4.35 (1-15)	0.064
Number of tumor nodules			
Solitary	51	138	0.318
Multiple	21	78	
Treatment			
Resection	26	78	-
Ablation Tx	23	69	
TACE	26	78	
AJCC 8th edition			
Stage Ⅰ	41	123	-
Stage Ⅱ	9	27	
Stage Ⅲ	22	66	
Child-Pugh grade			
A	67	171	-
B	14	42	
C	1	3	

PTA: Prothrombin time activity; AST: Aspartate aminotransferase (Normal range 15-40 U/L); ALT: Alanine aminotransferase (Normal range 9-50 U/L); TBil: Total billirubin (Normal range 5.1-19.0 μmol/L); AFP: Alpha fetoprotein (Normal range < 15 ng/mL); TACE: Transhepatic arterial chemotherapy and embolization.



	Seroclearance (n = 72)	Non-seroclearance (n = 216)
Follow-up duration (mo)[median (range)]	20 (2-120)	33 (2-119)
Overall survival rate		
1-yr	82.1%	85.1%
3-yr	43.2%	56.8%
5-yr	27.0%	45.2%

Figure 1 Overall survival of all hepatocarcinoma patients.

DISCUSSION

In the present study, through strict design matching, important confounding factors closely related to survival in patients with liver cancer (such as tumor stage, Child-Pugh score, and treatment method) were eliminated. Then, the effects of HBsAg seroclearance on the clinical characteristics and survival outcome of patients with hepatitis B-related liver cancer were determined. Patients with HBsAg seroclearance accounted for 5.14% (91/1772) of all cases of hepatitis B-related liver cancer in this study. Compared with the NSC group, the patients in the SC group were older and had higher platelet counts, but the overall prognosis of these patients was

Table 2 Baseline clinicopathological characteristics for the resection group

Variables	Seroclearance (n = 26)	Non-seroclearance (n = 78)	P value
Ages (median)	61.6 ± 10.1	56.4 ± 8.4	0.010
Sex (male:female)	22:6	69:9	0.216
Hemoglobin (mg/dL)	138.3 ± 21.0	142.1 ± 18.7	0.385
Platelet count (× 10^9/μL)	197.7 ± 91.6	154.8 ± 81.7	0.026
PTA (%)	96.9 ± 16.2	94.5 ± 17.8	0.538
Albumin (g/L)	41.8 ± 4.3	42.2 ± 4.8	0.716
AST (IU/L)	37.7 ± 39.5	42.7 ± 33.9	0.529
ALT (IU/L)	41.4 ± 41.6	41.2 ± 46.7	0.985
TBil (μmol/L)	19.7 ± 23.2	17.4 ± 14.8	0.570
AFP (ng/mL)			
⩽ 15	12	30	0.581
15-200	5	23	
⩾ 200	9	25	
Size of tumor (cm) [median (range)]	7.2 (2.3-15.4)	6.1 (2.3-15)	0.140
Number of tumor nodules			
Solitary	20	49	0.235
Multiple	6	29	
Pathological type			
HCC	18	74	0.002
ICC	3	2	
CHC	5	2	
Cell differentiated degree			
Highly differentiated	5	13	0.180
Moderately differentiated	11	48	
Poorly differentiated	10	17	
Liver cirrhosis			
Yes	20	68	0.221
No	6	10	
AJCC 8th edition			
Stage Ⅰ	14	42	-
Stage Ⅱ	2	6	
Stage Ⅲ	10	30	
Child-Pugh grade			
A	25	75	-
B	1	3	
C	0	0	

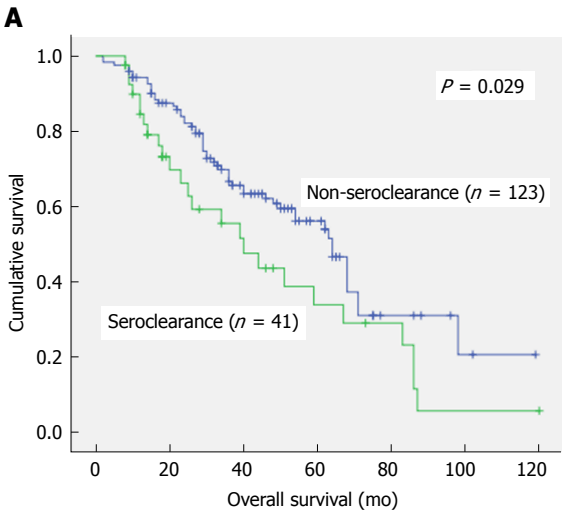
PTA: Prothrombin time activity; AST: Aspartate aminotransferase (Normal range 15-40 U/L); ALT: Alanine aminotransferase (Normal range 9-50 U/L); TBil: Total billirubin (Normal range 5.1-19.0 μmol/L); AFP: Alpha fetoprotein (Normal range < 15 ng/mL); HCC: Hepatocellular carcinoma; ICC: Intrahepatic cholangiocarcinoma; CHC: Combined hepatocellular carcinoma and cholangiocarcinoma.

worse.

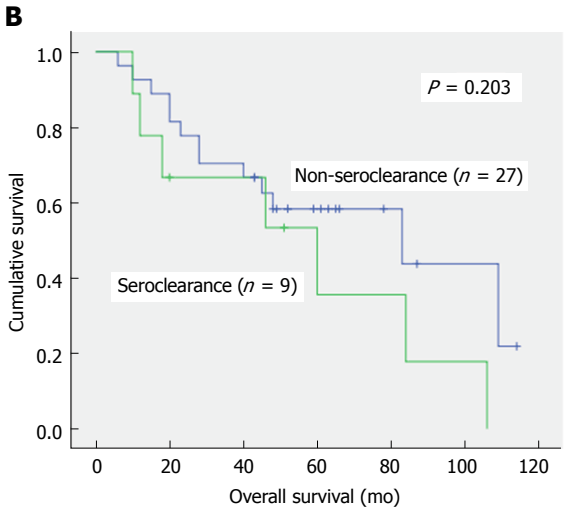
Previous studies have shown that the older the liver cancer patient with HBsAg seroclearance, the better the liver function reserve[6,9,14,15]. In the present study, this notion was confirmed by both holistic and subgroup analysis, and patients in the SC group were significantly older. These results indicated a delay in the occurrence of liver cancer, suggesting the protective effects of HBsAg seroclearance. As both groups were matched for Child-Pugh scores, we could not compare liver function between the two groups. However, a significant difference in platelet levels was found between the two groups. Platelet count is a sensitive index of hypersplenism, which indirectly reflects the severity of portal hypertension in cirrhosis. Our results showed that the higher the platelet count, the better the liver function reserve as in previous studies. This also suggests a potential protective effect of HBsAg seroclearance in the liver.

Although the above results confirmed liver protection by HBsAg seroclearance in patients with chronic hepatitis B, the final survival analysis yielded opposite data. Indeed, the prognosis of patients in the SC group was worse than that of the NSC group. Further analysis showed that for liver cancer patients with AJCC stage Ⅰ disease, the SC group showed worse overall survival compared with the NSC group. Generally, tumors at the early stage are more likely to be curatively treated with better therapeutic efficacy. Therefore, prognosis of these patients could better reflect the biological behavior of the tumor. Based on this common knowledge, the survival results in stage Ⅰ patients indicated the higher malignancy of tumors in the SC group. Although subsequent survival analysis of AJCC stage Ⅱ and Ⅲ patients showed no statistical significance, the SC group still showed a worse survival trend.

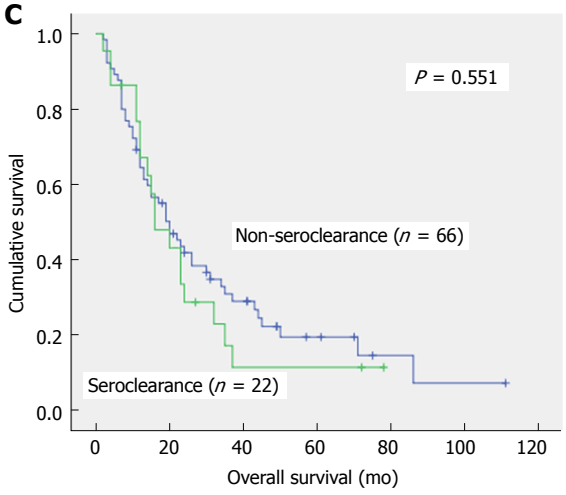
Comparing the pathological types of liver cancer treated with surgery, the two patient groups showed different distributions. The SC group showed more ICC and CHC cases, and malignancy in these two pathological types is obviously higher than that of HCC[16]. Unfortunately, the ablation group was limited by the rate of biopsy in addition



	Seroclearance (n = 41)	Non-seroclearance (n = 123)
Follow-up duration (mo) [median (range)]	20 (8-120)	37 (2-119)
Overall survival rate		
1-yr	84.60%	93.70%
3-yr	53.60%	79.30%
5-yr	33.80%	66.10%



	Seroclearance (n = 9)	Non-seroclearance (n = 27)
Follow-up duration (mo) [median (range)]	46 (10-106)	48 (6-114)
Overall survival rate		
1-yr	77.8%	89.5%
3-yr	57.3%	67.7%
5-yr	35.6%	52.1%



	Seroclearance (n = 22)	Non-seroclearance (n = 66)
Follow-up duration (mo) [median (range)]	16 (2-78)	19 (3-111)
Overall survival rate		
1-yr	67.2%	65.1%
3-yr	17.3%	31.5%
5-yr	11.5%	18.4%

Figure 2 Stratification analysis was carried out according to tumor stage. A: Overall survival of stage Ⅰ hepatocarcinoma (HCC) patients. B: Overall survival of stage Ⅱ HCC patients. C: Overall survival of stage Ⅲ HCC patients.

to the limitations of puncture pathology itself. Therefore, insufficient data could not be effectively analyzed. Results of the sole operation group could not represent the tumor type characteristics of the whole cohort. However, due to the highly significant difference observed in pathological types for the surgery group, the results were representative. More importantly, these findings corroborated survival data, which could partly explain the worse prognosis of patients with liver cancer in the SC group.

Studies have confirmed that HBV infection is closely related not only to HCC, but also to the incidence and prognosis of ICC[17-19]. In addition, a recent meta-analysis assessing the correlation between the HBV infection status and the risk of cholangiocellular carcinoma showed that consistent with positive HBsAg, HBsAg seroclearance is also a high risk factor for ICC[20]. This was confirmed by the above pathological findings. In another report, 928 patients with hepatitis B-associated ICC were assessed, including 24.7% with chronic hepatitis B and HBsAg seroclearance, and a high incidence of ICC in the SC group was also obtained[21]. Another important surgical and pathological finding in this study was the frequent occurrence of CHC in the SC group. Among twenty-six patients that underwent surgical excision in the SC group, five CHC cases were found, including two also diagnosed with cirrhosis. This pathological type is infrequently represented among all liver cancer cases, accounting for only 1.0%-4.7%. However, its malignancy exceeds that of ICC and HCC[16,22,23]. Zhou *et al*[24] found that HBV infection is an independent risk factor for CHC, with 64.3% of cases also diagnosed with cirrhosis. Multiple studies have shown that this mixed hepatocarcinoma might be largely derived from hepatic precursor cells (HPCs). Indeed, carcinogenic factors affect the multi-differentiation potential of HPCs, leading to the formation of a mixed source of hepatocytes and cholangiocytes[25-27]. Although previous studies have confirmed the associations of HBV infection with ICC and CHC incidence rates, it is very challenging to understand why these two highly malignant tumors were more likely to occur in the SC group. These findings still require confirmation in large sample trials.

The limitations of the present study should be mentioned. First, this was a single center retrospective case control study, with a possibility of selection bias in the control group. Second, since liver cancer incidence in patients with HBsAg seroclearance is low, the sample size was limited, and the number of cases after stratification was even more reduced, which resulted in wide confidence intervals. Third, because of difficulties in tracing the disease history, the current study lacked key data such as time of antiviral treatment, time of HBsAg seroclearance, and time of cirrhosis diagnosis. The correlation of HBsAg seroclearance with cirrhosis and liver cancer occurrence could not be well explained. Multicenter prospective studies are needed to solve the above problems.

In conclusion, patients with HBsAg seroclearance still have a risk of developing liver cancer. Although HBsAg seroclearance has some protective effects in respect to tumorigenesis, cirrhosis, and portal hypertension, prognosis in these patients is worse, likely because of the frequent occurrence of highly malignant ICC and CHC.

ARTICLE HIGHLIGHTS

Research background

Hepatitis B surface antigen (HBsAg) seroclearance is considered the gold standard for hepatitis B virus (HBV) clearance and chronic hepatitis B cure. Existing studies have confirmed that HBsAg seroclerance could result in significantly improved liver histology and biochemistry in patients with chronic HBV, but a certain incidence of liver cancer still exists. The current question is whether HBsAg seroclerance plays a role in the prognosis of liver cancer patients. Due to the small number of such patients, there is currently no reliable research and conclusion.

Research motivation

The effect of HBsAg seroclearance on prognosis in hepatitis B-related primary liver cancer remains unclear. The solution to this problem would improve the understanding of the etiology and pathogenesis of HBV-related liver cancer and to guide clinical treatment.

Research objectives

Our objective was to assess the impact of HBsAg seroclearance on survival outcomes in hepatitis B-related primary liver cancer.

Research methods

We designed a case-control study. Information from patients with hepatitis B-related liver cancer admitted to our hospital in 2008-2017 was retrieved. Cases diagnosed with HBsAg (-) andHBcAb (+) liver cancer were included in the HBsAg seroclearance (SC) group. HBsAg (+) liver cancer patients were strictly matched for liver cancer stage (AJCC staging system, 8th edition), Child-Pugh score, and first diagnosis/treatment method (surgery, ablation and TACE) and were assigned to the HBsAg non-seroclearance (NSC) group. Then, clinical, pathological, and survival data in both groups were assessed.

Research results

The SC and NSC groups comprised of 72 and 216 patients, respectively. Patient age ($P < 0.001$) and platelet count ($P = 0.001$) in the SC group were significantly higher than those of the NSC group. SC group patients who underwent surgery had more intrahepatic cholangiocarcinoma (ICC) and combined HCC-CC (CHC) cases than the NSC group, but no significant differences in tumor cell differentiation and history of liver cirrhosis were found between the two groups. Overall survival was lower in the SC group than the NSC group ($P = 0.019$). Survival of patients with AJCC stage I disease in the SC group was lower than that of the NSC group ($P = 0.029$).

Research conclusions

Seroclearance in patients with hepatitis B-related primary liver cancer has protective effects with respect to tumorigenesis, cirrhosis, and portal hypertension but confers worse prognosis, likely due to the frequent occurrence of highly malignant ICC and CHC.

Research perspectives

This is the first reliable study about the effect of HBsAg seroclearance on the prognosis of hepatitis B-related liver cancer. In the future, more SC patients should be accumulated, and it is necessary to further verify this result by propensity score matching.

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ORIGINAL ARTICLE

Retrospective Study

Machine learning to relate PM2.5 and PM10 concentrations to outpatient visits for upper respiratory tract infections in Taiwan: A nationwide analysis

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Abstract

AIM

To examine the accuracy of machine learning to relate particulate matter (PM) 2.5 and PM10 concentrations to upper respiratory tract infections (URIs).

METHODS

Daily nationwide and regional outdoor PM2.5 and PM10 concentrations collected over 30 consecutive days obtained from the Taiwan Environment Protection Administration were the inputs for machine learning, using multilayer perceptron (MLP), to relate to the subsequent one-week outpatient visits for URIs. The

URI data were obtained from the Centers for Disease Control datasets in Taiwan between 2009 and 2016. The testing used the middle month dataset of each season (January, April, July and October), and the training used the other months' datasets. The weekly URI cases were classified by tertile as high, moderate, and low volumes.

RESULTS

Both PM concentrations and URI cases peak in winter and spring. In the nationwide data analysis, MLP machine learning can accurately relate the URI volumes of the elderly (89.05% and 88.32%, respectively) and the overall population (81.75% and 83.21%, respectively) with the PM2.5 and PM10 concentrations. In the regional data analyses, greater accuracy is found for PM2.5 than for PM10 for the elderly, particularly in the Central region (78.10% and 74.45%, respectively), whereas greater accuracy is found for PM10 than for PM2.5 for the overall population, particularly in the Northern region (73.19% and 63.04%, respectively).

CONCLUSION

Short-term PM2.5 and PM10 concentrations were accurately related to the subsequent occurrence of URIs by using machine learning. Our findings suggested that the effects of PM2.5 and PM10 on URI may differ by age, and the mechanism needs further evaluation.

Key words: Particulate matter 2.5; Particulate matter 10; Upper respiratory infections; Machine learning; Air pollution

Core tip: Particulate matter (PM) 2.5 and PM10 air pollutants can trigger inflammation and predispose the respiratory tract to infections. This study used the multilayer perceptron (MLP) machine learning architecture to relate the daily PM2.5 and PM10 concentrations over 30 consecutive days to the subsequent one-week outpatient visits for upper respiratory tract infections (URIs) in Taiwan between 2008 and 2016. In the nationwide data analysis, PM2.5 and PM10 concentrations can precisely predict the volumes of URI for the elderly (89.05% and 88.32%, respectively) and the overall population (81.75% and 83.21%, respectively). Our findings suggested that machine learning could accurately relate PM2.5 and PM10 concentrations to the outpatient visits for URI, especially for the elderly population.

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INTRODUCTION

Particulate matter (PM) 2.5 and PM10, also known as particle pollutions, refer to a mixture of liquid droplets and solid particles in the air with diameters $\leqslant$ 2.5 μm and $\leqslant$ 10 μm, respectively. In developing countries, PM2.5 accounts for half of PM10 concentrations, whereas in developed countries, PM2.5 is estimated to account for 50%-80% of the PM10 concentrations[1]. Both PM2.5 and PM10 can deposit in the respiratory tract and may trigger inflammatory reactions that increase the plasma interleukin-6 and fibrinogen levels[2]. The inflammation process related to air pollution might decrease innate immunity and predispose robust individuals to acute illnesses, such as upper respiratory tract infections (URIs), and the development of chronic disease, such as lung malignancies[3-5]. Several observational studies have revealed that PM2.5 and PM10 concentrations may be associated with the occurrence of URIs[5,6] and may increase the risk of mortality related to hospitalized pneumonia[7].

Machine learning utilizes computational statistics to explore optimized algorithms, which can learn from and make predictions based on data. Machine learning for potentially hazardous exposure has been successfully applied to predict the occurrence of several clinical diseases, such as myocardial infarction, and the risk of mortality in previous studies[8]. In addition, machine learning, such as artificial neural networks, can provide us with an opportunity for big data training for the prediction of clinical diseases[9]. For example, some models using convolutional neural networks for training with hundreds of thousands of fundus images to predict the presence and the severity of diabetic retinopathy have been well established[10-13]. Carnegie Mellon's Delphi group of the United States Centers of Disease Control has been working to create a machine learning model that accurately tracks the spread of the flu[14].

Since the severity of air pollution varies geographically, the hazardous effect on human health may also differ by region and ethnicity. It is reasonable to create a surveillance system for forecasting the probability of disease occurrence related to regional air pollution. Multilayer perceptron (MLP) artificial intelligence, a type of machine learning similar to the human neural network, is formed by at least three layers of nodes that make use of nonlinear activation for data training[15]. Accordingly, we attempted to establish such an MLP model to relate PM2.5 and PM10 concentrations to the volume of outpatient visits for acute URIs in Taiwan.

MATERIALS AND METHODS

Data collection

The datasets of outpatient visits for URIs were obtained from the website of the Centers for Disease Control (CDC) of Taiwan for the period from January 2009 to December 2016, which is 417 wk in total. Clinical

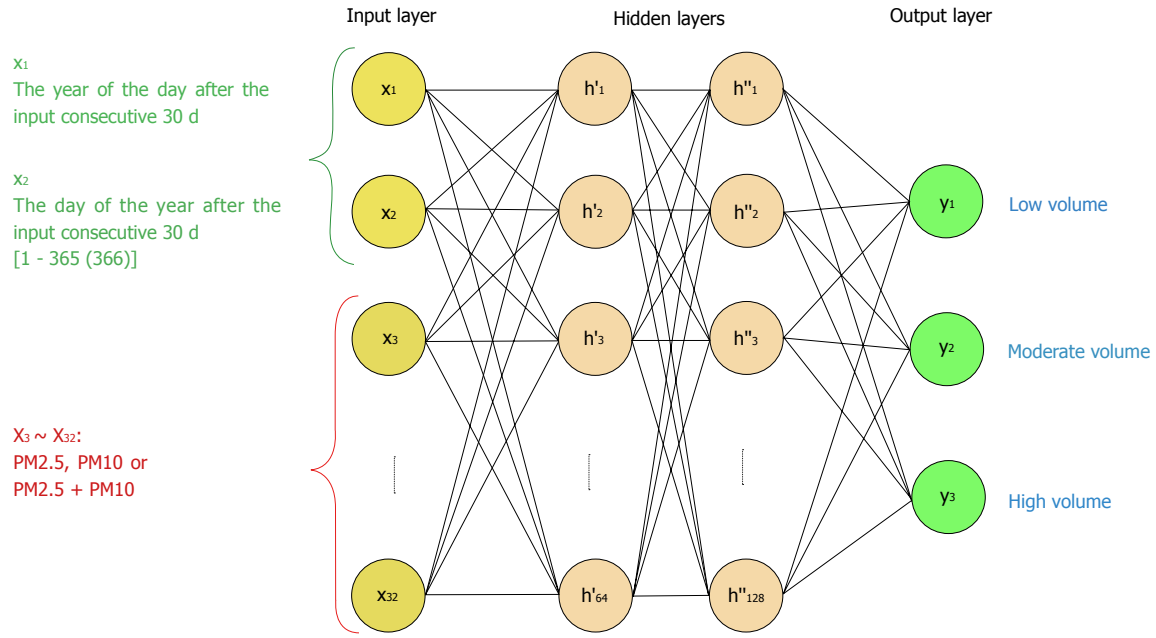

Figure 1 Multilayer perceptron model for the proposed algorithm.

physicians have to diagnose URIs for patients according to clinical symptoms, physical presentations, and objective laboratory data at an outpatient department. The cases of URIs were retrieved from the Taiwan Nationwide Health Insurance records, which is based on the International Classification of Diseases, Ninth Revision, Clinical Modification (ICD-9-CM) codes 465, 487.1, 488.02, 488.01 and 034.0 for acute URIs (Supplemental Table 1). The datasets of PM2.5 and PM10 were obtained from the Taiwan Environment Protection Administration, and the PM concentrations were measured and collected outdoors from 55 ambient air quality monitoring sites spread throughout Taiwan.

MLP model

Figure 1 shows the MLP architecture with a forward and backward propagation learning algorithm to accurately relate PM2.5 and PM10 concentrations to the occurrence of outpatient visits for acute URI, which is classified by tertile as high, moderate, and low volumes. The testing used the middle month datasets of each season in Taiwan, which account for 33% of all datasets [January (winter), April (spring), July (summer), and October (fall)], and the training used the other months' datasets, which account for 67% [December and February (winter), March and May (spring), June and August (summer), and September and November (fall)]. The training and testing procedures were repeated multiple times to determine optimal outcomes.

Statistical analysis

Daily average PM2.5 and PM10 concentrations for 30 consecutive days were used as the inputs of the MLP model to relate to the outputs of subsequent one-week URI volumes. PM2.5 and PM10 concentrations were normalized before inputting them into the MLP model. Based on the criteria of the Taiwan Environment Protection Administration in Supplemental Table 1, the hazardous to human health cut-off levels of PM2.5 and PM10 were $\geqslant$ 250.4 μg/m^3 and $\geqslant$ 424.0 μg/m^3, respectively, which were set as 1[16]. In addition, the cut-off levels of PM2.5 and PM10 suggestive of good air quality were $\leqslant$ 15.4 μg/m^3 and $\leqslant$ 54.0 μg/m^3, respectively, which were set as 0. The PM values within the upper and lower cut-off levels were normalized to between 0 and 1 (Supplemental Table 2). If both PM2.5 and PM10 were treated as the inputs together in the MLP model, the normalization of PM2.5 + PM10 would be the average of the sum of the normalizations of PM2.5 and PM10. One-week volumes of URIs were used as outputs because of the time lag effect[5], since ill patients may seek a medical consultation days after the beginning of the infection, when the symptoms have worsened. Additionally, the accuracy of MLP machine learning for the overall and elderly ($\geqslant$ 65 years) patients was estimated. The MLP model was tested in a nationwide data analysis and in several regional data analyses of western Taiwan consisting of the Northern (business and economic areas), Central, and Southern regions (industrial areas), and eastern Taiwan, which is represented by the Eastern region (a national park area) (supplemental Figure 1).

RESULTS

Figure 2 shows the average daily concentrations of PM2.5 and PM10 from December 2008 to December 2016 and the average numbers of outpatient visits

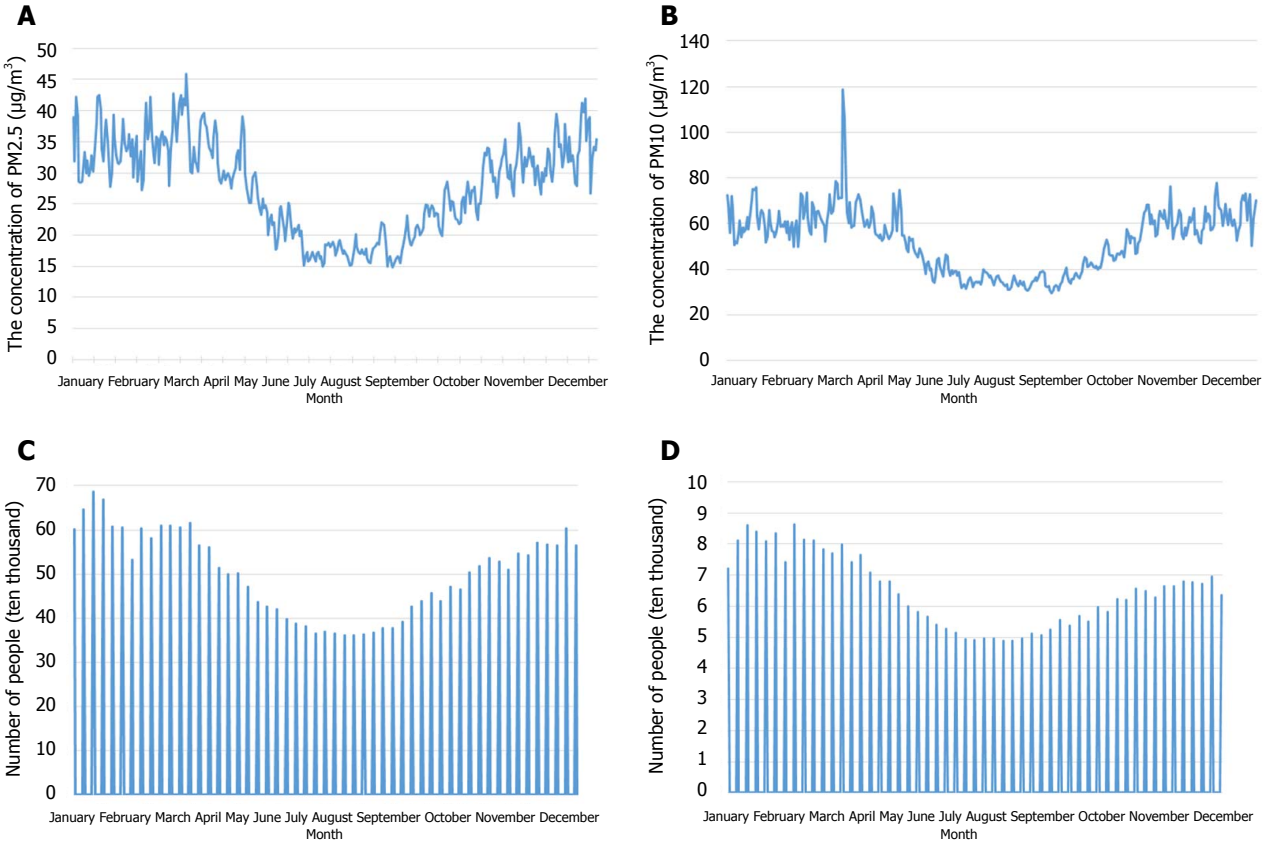

Figure 2 The average daily concentrations and weekly numbers of outpatient visits for upper respiratory tract infections in each month. A and B: PM2.5 and PM10, repectively (December 2008 - December 2016); C and D: The overall and the elderly patients, repsectively (January 2009 - December 2016). PM: Particulate matter.

for URIs for the overall population and the elderly population in each month from January 2009 to December 2016. As shown, the concentrations of PM2.5 and PM10 distribute as a diurnal curve and peak in the winter and spring seasons. The PM2.5 concentrations were between 15 μg/m^3 and 46 μg/m^3, and the PM10 concentrations ranged from 30 μg/m^3 to 100+ μg/m^3. The occurence of total and elderly outpatient visits for acute URI are most prevalent in winter and spring as well, which is correlated with the PM2.5 and PM10 levels. With regard to the volume of outpatient visits for URIs, the number of monthly overall URI cases ranges from 35000 to 70000, and the number of monthly elderly URI cases ranges from 4800 to 9000.

Table 1 reveals the averge PM2.5 and PM10 concentrations and the numbers of outpatient visits for URIs in each season by the four regions and in all of Taiwan. In general, the regional patterns of PM concentrations were in line with the nationwide pattern in Taiwan. On average, the Eastern region, followed by the Northern region, had the lowest PM concentrations. Conversely, the Central and Southern regions, consisting of many of the industrial counties of Taiwan, had the highest PM concentrations. Similarly, the regional patterns of outpatient numbers were in line with the nationwide pattern. The Nothern region had the greatest numbers of URIs and the Eastern region had the least numbers of URIs. The URI prevalence was mostly higher in the winter and spring.

Table 2 demonstrates the nationwide and regional data analysis results of the accuracy of MLP machine learning to relate PM2.5 and PM10 concentrations to the volume of outpatient visits for URIs in the overall population and the elderly population. The nationwide data analysis reveals that PM2.5 and PM10 concentrations can correctly relate to the volumes of URI in the elderly population (89.05% and 88.32%, respectively) and the overall population (81.75% and 83.21%, respectively). In the regional analyses, PM2.5 and PM10 concentrations have the greatest accuracy for the elderly population in the Eastern and Northern regions (80.29%/81.75% and 80.43%/76.81%, respectively), which are the two least air polluted areas in Taiwan. By contrast, the accuracy of URI occurrence based on large data mining of PM2.5 and PM10 in all regions of Taiwan is relatively lower at approximately 63% to 73% for the overall population. In addition, PM2.5 has greater accuracy than PM10 for the elderly, particularly in the Central region (78.10% and 74.45%, respectively), whereas PM10 has greater accuracy than PM2.5 for the overall population, particularly in the Northern region (73.19% and 63.04%, respectively). Notably, the MLP performance was better at a nationwide scale than that at the regional scale. When the

Table 1 Nationwide and regional average particulate matter concentrations between December 2008 and December 2016 and the number of outpatient visits for upper respiratory infections in each season in Taiwan from January 2009 to December 2016

Regions/PM (μg/m3)		Spring	Summer	Fall	Winter
Taiwan	PM2.5	30.90 ± 12.30	17.97 ± 6.37	28.00 ± 10.20	34.44 ± 13.10
	PM10	58.05 ± 27.47	35.00 ± 7.74	53.96 ± 17.46	62.43 ± 21.31
Northern region	PM2.5	27.37 ± 12.43	18.63 ± 7.43	20.36 ± 10.02	25.95 ± 13.78
	PM10	49.52 ± 33.52	33.30 ± 11.64	38.63 ± 18.51	46.44 ± 24.17
Central region	PM2.5	35.36 ± 16.13	20.12 ± 9.37	32.99 ± 13.90	36.37 ± 15.64
	PM10	60.55 ± 30.03	35.64 ± 11.56	57.15 ± 20.32	60.30 ± 23.70
Southern region	PM2.5	34.99 ± 17.46	17.32 ± 8.71	37.33 ± 15.42	49.14 ± 15.95
	PM10	66.75 ± 32.10	36.51 ± 11.21	70.60 ± 25.93	87.42 ± 24.73
Eastern region	PM2.5	15.37 ± 8.05	10.36 ± 4.94	13.49 ± 7.82	15.62 ± 8.73
	PM10	30.49 ± 15.45	24.04 ± 9.86	31.85 ± 23.11	30.12 ± 14.67
Regions/URI patients (× 10^3)					
Taiwan	Overall	523.75 ± 93.61	375.67 ± 35.07	492.46 ± 63.48	607.31 ± 124.83
	Elderly	70.13 ± 11.27	50.62 ± 4.75	60.98 ± 6.91	77.52 ± 17.23
Northern region	Overall	210.90 ± 35.83	148.45 ± 14.46	192.93 ± 26.09	238.55 ± 53.95
	Elderly	25.10 ± 3.99	17.73 ± 1.65	20.93 ± 2.55	27.19 ± 6.71
Central region	Overall	102.77 ± 20.04	72.74 ± 7.30	97.90 ± 12.79	121.29 ± 24.65
	Elderly	13.53 ± 2.23	9.63 ± 0.94	11.85 ± 1.36	14.88 ± 3.27
Southern region	Overall	77.95 ± 14.09	62.25 ± 7.06	78.97 ± 10.53	95.38 ± 18.11
	Elderly	11.78 ± 1.91	9.57 ± 1.17	11.29 ± 1.33	13.74 ± 2.78
Eastern region	Overall	12.91 ± 2.22	8.71 ± 1.12	11.41 ± 1.63	14.35 ± 2.67
	Elderly	2.29 ± 0.34	1.52 ± 0.18	1.86 ± 0.24	2.51 ± 0.54

PM: Particulate matter; URI: Upper respiratory infection.

Table 2 The accuracy of Particulate matter machine learning for PM2.5 and PM10 concentrations to predict the events of outpatient visits for upper respiratory infections by the four regions and in all of Taiwan

Accuracy (%)		Overall population	Elderly population
Taiwan	PM2.5	81.75	89.05
	PM10	83.21	88.32
	PM2.5 + PM10	83.21	89.05
Northern region	PM2.5	63.04	80.43
	PM10	73.19	76.81
	PM2.5 + PM10	65.94	78.99
Central region	PM2.5	69.34	78.10
	PM10	72.26	74.45
	PM2.5 + PM10	69.34	77.37
Southern region	PM2.5	71.01	76.09
	PM10	71.74	74.64
	PM2.5 + PM10	71.74	74.64
Eastern region	PM2.5	67.15	80.29
	PM10	71.53	81.75
	PM2.5 + PM10	71.53	84.67

PM: Particulate matter.

PM2.5 and PM10 concentrations were combined in the MLP model, the accuracy was not improved much for either the elderly or the overall population.

DISCUSSION

In previous studies, the hazardous effect of high levels of PM2.5 and PM10 exposures on the occurrence of URIs had been observed[2,5]. These studies often applied a case-crossover design using the case PM data on the event day of disease occurrence to compare with other control PM data on prospective and retrospective days to see the odds ratio of URIs related to the high PM air pollutions. To our best knowledge, the effect of PM on the respiratory tract may be synergic or cumulative, and using one-day PM concentrations to estimate the URI risk could have bias. Our study used a novel procedure of MLP machine learning, which can process large amounts of successive 30-d PM concentration data from nationwide and regional perspectives to relate to the URI volumes, which would improve the solidity of the relationship.

We found that the concentrations of PM2.5 and PM10 peak in the winter and spring, which could be highly related to several meteorological parameters, such as gravity, outdoor temperature, humidity, wind speed, and rain[17,18]. The URI occurrence may be associated with factors by which the pathogens can grow

rapidly and predispose robust individuals to acute illness. Higher PM levels coinciding with the pathogens' active seasons might contribute to a high prevalence of URIs. In addition, we also showed that the relationship of PM concentrations with acute URI had the best result for the elderly population. This could be explained in part by the fact that the elderly, who had many comorbidities and weaker immunity, were more likely to have acute illness when exposed to multiple air pollutions.

Another important finding was that the MLP models for the PM2.5 and PM10 training data to relate the concentrations of PM2.5 and PM 10 to URI occurrence were more accurate in areas of low air pollution for the elderly. It is reasonable that although PM air pollutants are hazardous to human health, several other toxins, such as sulfide dioxide (SO_2) and carbon monoxide (CO), could also contribute to the development of URIs in areas of heavy air pollution[19]. As a result, the importance of PM on the occurrence of URI might be attenuated if there were many other coexisting air pollutants. In addition, the MLP machine learning more accurately related PM2.5 to the URI volumes for the elderly, whereas the accuracy with PM10 was better for the overall population. Further evaluations are still needed to determine if the effect of PM2.5 and PM10 on the respiratory tract may differ by age, which influences the physical activity and outdoor exposure time. Moreover, there was no additional predictive benefit of putting both PM concentrations together into the MLP models. This was likely because PM2.5 is included in PM10 and they are highly correlated or because of the effect of overfitting in the machine learning process[20].

Notably, the MLP models utilized for the big PM data training had the greatest accuracy at the nationwide scale. This could be because more PM data were involved in the nationwide scale, which facilitated the machine learning and possibly led to the greater accuracy. However, the explanation did not hold true with regard to the regional difference. For instance, the Northern region has the largest population in Taiwan but, paradoxically, yields the lowest accuracy. In contrast, the Eastern region has the smallest population in Taiwan but shows the highest accuracy. Therefore, we speculate that many factors other than sample size, such as the severity of air pollution or the heterogeneity of unrecognized factors, such as migration, may affect the results of the MLP.

Our study has several strengths. First, the PM2.5 and PM10 concentrations as well as the diagnosis of URI were reliable and objectively retrieved from government agencies. Second, a large amount of data from the publicly available website could be easily utilized for ongoing studies, and the results are reproducible. On the other hand, although the MLP is a well-known machine learning method, there are a few limitations in our study. First, we used only PM concentration data in this study, and we may need data for more air pollutants, such as SO_2 and CO, and other meteorological parameters for further adjustments of the study. Second, details of the baseline characteristics of the patients with URIs, such as sex, body weight, and underlying comorbidities, were lacking, and the results were mainly based on the assumption that all people did not migrate frequently during the study period, which may result in potential bias if the assumptions were inaccurate. Third, we could not provide direct evidence for the cause-effect relationship between PM and acute URIs, which might be due to coincidence merely based on the retrospective nature of the study design.

In conclusion, MLP machine learning could accurately relate short-term PM2.5 and PM10 concentrations to subsequent outpatient visits for URIs. Our findings suggested that the elderly population and areas with less air pollution may have better MLP test results. In addition, the hazardous effect of PM2.5 and PM10 on URIs may differ by age, which is possibly related to daily activity and outdoor exposure time, which needs further evaluation. We also noticed that the performance of the MLP at the nationwide scale was better than that at the regional scale. Whether this finding was because of a larger population sample size or a higher heterogeneity in the nationwide scale is unknown, and this also requires further investigation.

ARTICLE HIGHLIGHTS

Research background

PM2.5 and PM10, also known as particle pollutions, can deposit in the respiratory tract and may trigger inflammatory reactions. Several studies have revealed that PM2.5 and PM10 concentrations may be associated with the occurrence of upper respiratory tract infections (URIs) and increase the mortality related to hospitalized pneumonia. Machine learning utilizes computational statistics to explore optimized algorithms that can learn from and make predictions based on data. Machine learning for potential hazardous exposures has been successfully applied to predict the occurrence of several clinical diseases, such as myocardial infarction, and the related risk of mortality. In addition, machine learning, such as artificial neural networks, can provide us an opportunity for big data training for the prediction of clinical diseases. For example, Carnegie Mellon's Delphi group of the United States Centers of Disease Control has been working to create a machine learning model that accurately tracks the spread of the flu.

Research motivation

Since the severity of air pollution varies geographically, the hazardous effect on human health may also differ by region and ethnicity. It is reasonable to create a surveillance system to forecast the probability of disease occurrence related to regional air pollution. Accordingly, we attempted to establish a model of machine learning to relate PM2.5 and PM10 concentrations to the volume of outpatient visits for acute URIs in Taiwan.

Research objectives

To examine the accuracy of machine learning to relate PM2.5 and PM10 concentrations to URIs.

Research methods

Daily nationwide and regional outdoor PM2.5 and PM10 concentrations collected over 30 consecutive days from the Taiwan Environment Protection Administration were the inputs for the multilayer perceptron (MLP) machine learning to relate to the subsequent one-week outpatient visits for URIs. The URI data were obtained from the Centers for Disease Control datasets in

Taiwan between 2009 and 2016. The testing used the middle month dataset of each season (January, April, July, and October), and the training used the other months' datasets. The weekly URI cases were classified by tertile as high, moderate, and low volumes.

Research results

Both PM concentrations and URI cases peak in the winter and spring. In the nationwide data analysis, MLP machine learning can accurately relate PM2.5 and PM10 concentrations with the URI volumes of the elderly (89.05% and 88.32%, respectively) and the overall population (81.75% and 83.21%, respectively). In the regional data analyses, PM2.5 has greater accuracy than PM10 for the elderly, particularly in the Central region (78.10% and 74.45%, respectively), whereas PM10 has greater accuracy than PM2.5 for the overall population, particularly in the Northern region (73.19% and 63.04%, respectively).

Research conclusions

Machine learning could accurately relate short-term PM2.5 and PM10 concentrations to subsequent URI occurrence. Our findings suggested that the effects of PM2.5 and PM10 on URI may differ by age, and the mechanism needs further evaluation.

Research perspectives

We used MLP machine learning to successfully relate PM concentrations data to the volume of URI cases. Data for more air pollutants and other meteorological parameters can be applied to the current MLP model in future work.

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ORIGINAL ARTICLE

Clinical Trials Study

Combined exercise improves gastrointestinal motility in psychiatric in patients

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Abstract

AIM

To examine the effect of combined exercise on colonic transit time (CTT) in admitted psychiatric patients.

METHODS

Over a 6-mo period, consecutive in patients with mental illness were recruited from the Somang Hospital Psychiatry Unit. A combined exercise program that included 60 min per day of exercise 3 d per week for 12 wk was performed. Physical fitness and CTT of the patients were measured twice before and twice after the exercise program. CTT was measured using a multiple marker technique with a radio-opaque marker. Changes in the exercising patients' CTT and weight-,

cardiovascular- and fitness-related parameters were statistically assessed.

RESULTS

After the 12-wk combined exercise intervention, decreased intestinal transit time was observed in all CTTs of the exercise group, including the right CTT (exercise: 15.6 ± 15.2 *vs* 9.2 ± 11.9, control: 13.1 ± 10.4 *vs* 10.9 ± 18.7), left CTT (exercise: 19.7 ± 23.5 *vs* 10.4 ± 13.2, control: 19.2 ± 19.0 *vs* 16.9 ± 19.8), recto-sigmoid CTT (exercise: 14.3 ± 16.7 *vs* 6.7 ± 7.9, control: 15.0 ± 14.4 *vs* 19.3 ± 30.3), and total colonic transit time (TCTT) (exercise: 50.2 ± 38.1 *vs* 27.1 ± 28.0, control: 47.4 ± 34.6 *vs* 47.3 ± 47.3). After the 12-wk combined exercise period, TCTT was significantly shortened in the exercise group compared with that in the control group. In addition to eating habits, water intake, and fiber intake, the increased physical activity level as a result of the 12-wk combined exercise program reduced the CTT.

CONCLUSION

The CTT of the psychiatric patients was reduced due to increased physical activity *via* a 12-wk combined exercise program.

Key words: Combined exercise; Constipation; Colonic transit time; Radio-opaque marker; Psychiatry unit patient

Core tip: Maintaining physical activity routine for in patients of closed wards in mental health facilities remains a major challenge. Long-term inactivity is a risk factor for decreased gastrointestinal motility, which leads to constipation, weight gain, and related metabolic and cardiovascular disorders and can affect drug absorption. In this study, implementation of a 12-wk combined exercise program was shown to be beneficial in reducing colonic transit time and increasing leg strength.

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INTRODUCTION

The estimated incidence of colorectal cancer according to the 2014 Cancer Statistics in the United States was 5.8% (96830 people), and the estimated mortality rate was 8.5% (50310 people) of overall cancer mortality[1]. Colon cancer is the third most common cancer and reportedly affected 9.7% (1.2 million people) of the world's population in 2008[2]. The risk factors for colon cancer can be divided into physiological factors, such as age and heredity, and lifestyle factors, such as smoking, drinking, and poor eating habits. Among the risk factors for several colon cancers, constipation is a concern in many countries and is a factor that can be reduced by lifestyle changes.

According to cohort research in 2010, 4176 people reportedly suffered from constipation[3]. Typical symptoms of constipation according to the Rome Criteria are excessive squeezing, hard stools, sensation of incomplete evacuation, sensation of anal closure, ongoing treatment for evacuation, and an abnormal number of evacuations (less than 3 times a week)[4]. Constipation is diagnosed when two or more symptoms are concurrent. In addition, constipation types are classified into "slow colonic transit" and "pelvic floor dysfunction" categories[5].

"Slow colonic transit" refers to slow movement from the proximal colon to the distal colon and then to the rectum and is thought to be caused by poor dietary habits, cultural habits, and pathophysiology. The method for measuring "slow colonic transit" frequently involves a radio-opaque marker[6], and the results can form the basis of a constipation diagnosis; if the radioactive non-transmission marker remains in the colon for a long time (KolomarkTM, MJ Tech, Pyeongtaik Korea), then a decrease in colonic inertia and disordered evacuative function are indicated. Previous studies have shown that increased prevalence of constipation is correlated with decreased levels of physical activity (PA)[7-9].

Lower PA (*e.g.*, sitting, watching television) may increase the risk of low gastrointestinal (GI) motility, and colorectal cancer[10,11], obesity[12], and diabetes[13] on mentally ill patients in a closed ward. Promoting PA levels can be challenging for medical care system working with mentally ill patients in a closed ward.

Many studies have been conducted to identify strategies to reduce colonic transit time (CTT) and thus improve constipation, including changes in food intake and increasing PA[14-17].

Some studies have shown that increasing PA levels may help to short colon transit time and to prevent constipation on mentally ill patients. Recently, various studies have been conducted on the effects of combined exercise, which entails both aerobic and resistance exercises to maximize their effects. However, no studies have been conducted on the effect of combined exercise on mentally ill patients hospitalized in closed wards.

Although studies on aerobic exercise among the exercise therapies for colon peristalsis are increasing, research on the effect of combined exercise on constipation is limited. Therefore, we examined the effects of a combined exercise program on CTT and physical characteristics of mentally ill patients in a closed ward to provide basic evidence for prescribing a combined exercise program to patients alongside medication.

Table 1 Combined exercise program for 12 wk

Combined exercise program		
Frequency	60 min, 3 times/wk, 12 wk	
Exercise Program	Exercise (time): Contents	
Warm-up	Stretching (10 min): Various stretches	
Main exercise (40 min)	Resistance exercise[1] (20 min)	Aerobic exercise (20 min)
	Chest press Seated row Squat Shoulder press Bicep curl Tricep extension Calf raise Reverse crunch	Jogging/running
	10 reps, 2 sets	VO2max 60%
Cool-down (10 min)	Stretching (10 min): Various stretches	

[1]With Thera band.

MATERIALS AND METHODS

Participants

Consecutive male in patients with mental illness admitted for treatment in the closed ward of Somang Hospital Psychiatry Unit (Eumsung-gun, South Korea) over a 6-mo period were recruited to the study. Men with mental illness who did not participate in any regular exercise program over the past year were selected; the combined exercise group included 31 men, and the control group included 21 men. Among the forty men selected to participate in the combined exercise group, 9 men left the study due to personal illness, nonparticipation in exercise, or a change in medication. Among the thirty men selected to participate in the control group, 9 men left the study due to nonparticipation at the time of measurement, discontinuation of further exercise, or for personal reasons.

All subjects voluntarily provided informed written consent for the use of their data. Among all potential subjects, those with a restriction in normal PA, those with cardiovascular or orthopedic disease that could affect CTT, those who were unable to discontinue drugs due to functional stomach diseases, those who were on a prescription course of anti-constipation drugs, and those with diabetes mellitus or hypertension were excluded from the current analysis[18-20].

Combined training

All subjects in the exercise group were asked to perform a combined exercise program for 12 wk. Elastic band exercises and a running program were used for resistance exercise and aerobic exercise, respectively. The elastic band program based on the Thera-band manual was used to determine exercise intensity. The Thera-bands are color-coded to match various intensities (kg) and stretch lengths; according to the manual, the green band was used for this program. The first 2 wk served as an adjustment period; from the third week, the intensity increased from 10 RM to 15 RM with a 10-s rest period between each interval. According to the ACSM (2006) guidelines, aerobic exercise was regulated to achieve a maximum volume of oxygen (VO2 max) of 60% for target heart rate and was maintained using a Heart Rate Analyzer (Polar Electro OY, Finland). The combined exercise program is presented in Table 1.

Measurement of physical characteristics

Bioelectrical impedance analysis (Inbody 3.0, Biospase, South Korea) was used to measure height, weight, and body mass index (BMI). Blood pressure (BP) was measured using a sphygmomanometer (SPRIT CK-101, Sankei, Japan) in the supine position after a 5-min rest. Efforts were made to rule out any extrinsic factors that could affect blood pressure, such as temperature, degree of physical activity (PA), smoking, and diet[10,12]. Thigh circumference was measured as the distance around the fullest part of the thigh. Waist circumference was measured as the distance around the abdomen, just above the hip bones.

Measurement of fitness

Physical strength measurements included grip strength, leg strength, standing high jump, sit and reach, balance, and cardiopulmonary endurance. Grip strength was measured using a dynamometer (T.K.K 5401, Japan), leg strength was measured using a leg-extension machine (to measure isometric knee extensor force) (T.K.K 5710M, Japan), and vertical jump was measured using a Sargent jump measurement device (T.K.K 5406, Japan). The cardiopulmonary function evaluation was performed using the YMCA step test. Heart rate was measured during recovery after the subject stepped up and down repeatedly on a 30.5-cm high step box 24 times per minute to a metronomic beat of 96 beats per minute for 3 min[18,20].

Measurement of CTT

CTT was measured twice, after exercise and before exercise using a multiple marker technique with a radio-opaque marker. The subjects were given one gelatin capsule containing 20 radio-opaque markers at the same time every day for three days (Kolomark™, MI Tech, Pyeongtaik, South Korea). At the same time on days 4 and 7, supine abdominal radiography was performed. The mean CTT (in hours) was calculated by counting the number of radio-opaque markers remaining in the entire colon and in the segments of the colon and then multiplying this number by 1.2[6,8,21-23].

Statistical analysis

The study data, expressed as the means ± SD, were analyzed using SPSS PC+ for Windows, version 18.0 (SPSS Inc., Chicago, IL, United States). Changes in fitness and segmental colon transit time pre- and post-

Table 2 Changes in the physical characteristics of the subjects after 12 wk of combined exercise training

Variable	Combined exercise (n = 31)		Control (n = 21)		P
	Pre	Post	Pre	Post	
Age (yr)	48.8 ± 9.5		49.9 ± 11.2		
Height (cm)	165.6 ± 6.7	165.7 ± 6.6	168.2 ± 4.1	167.8 ± 5.2	0.804
Weight (kg)	70.9 ± 12.8	69.1 ± 11.3	68.6 ± 13.9	67.0 ± 14.3	0.961
BMI (kg/m^2)	25.8 ± 4.4	25.0 ± 3.9	24.1 ± 4.1	24.2 ± 4.7	0.557
Lean body mass (kg)	47.4 ± 7.1	49.2 ± 8.1	44.7 ± 5.2	46.8 ± 7.3	0.890
Body fat percentage (%)	30.7 ± 9.5	28.0 ± 9.3	33.4 ± 10.4	30.9 ± 9.2	0.913
SBP (mmHg)	122.0 ± 20.1	115.7 ± 15.4	125.9 ± 16.9	112.8 ± 19.3	0.075
DBP (mmHg)	79.3 ± 10.2	73.8 ± 9.2	87.1 ± 15.3	76.9 ± 13.1	0.154
Resting heart rate	88.1 ± 15.7	81.2 ± 11.9	87.4 ± 10.2	80.5 ± 8.5	0.980
Thigh circumference (cm)	42.6 ± 5.9	47.0 ± 11.4	44.0 ± 6.0	46.3 ± 8.5	0.496
Waist circumference (cm)	90.8 ± 12.2	87.5 ± 11.7	91.8 ± 12.7	90.5 ± 14.6	0.532

The values are shown as the mean ± SD. BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure.

Table 3 LChanges in the physical fitness of the subjects after 12 wk of combined exercise training

Variable	Combined exercise (n = 31)		Control (n = 21)		P
	Pre	Post	Pre	Post	
Grip strength (kg)	25.8 ± 9.3	28.0 ± 8.6	29.5 ± 10.0	29.3 ± 8.2	0.113
Leg strength (kg)	42.5 ± 21.0	62.6 ± 21.6[b]	42.4 ± 15.2	42.8 ± 5.5	0.009[d]
YMCA step test (beat per min)	122.4 ± 16.0	111.0 ± 13.9	120.3 ± 16.4	119.6 ± 11.7	0.084
Vertical jump (cm)	21.5 ± 10.1	27.8 ± 11.2[f]	21.5 ± 12.2	24.3 ± 11.2	0.159
Sit and reach (cm)	4.3 ± 8.9	5.7 ± 8.8[a]	3.9 ± 8.5	5.4 ± 8.1	0.549

The values are shown as the mean ± SD. The change in leg strength reflect the difference in the time × group interaction between the exercise and control groups ([d]P = 0.009); Significant differences between pre and post combined exercise group in leg strength ([b]P = 0.003). Significant differences between pre and post combined exercise group in vertical jump ([f]P = 0.001). Significant differences between pre and post combined exercise group in sit and reach ([a]P = 0.033).

exercise training were assessed by two-way repeated ANOVA. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Changes in the physical characteristics of the subjects after 12 wk of combined exercise training

The physical characteristics of the subjects are shown in Table 2. The subjects in the exercise group showed decreases in body fat percentage (baseline: 30.7 ± 9.5 *vs* study-end: 28.0 ± 9.3), systolic blood pressure (SBP) (122.0 ± 20.1 *vs* 115.7 ± 15.4), diastolic blood pressure (DBP) (79.3 ± 10.2 *vs* 73.8 ± 9.2), resting heart rate (88.1 ± 15.7 *vs* 81.2 ± 11.9), and waist circumference (90.8 ± 12.2 *vs* 87.5 ± 11.7); however, the patients showed increases in lean body mass (47.4 ± 7.1 *vs* 49.2 ± 8.1) and thigh circumference (42.6 ± 5.9 *vs* 47.0 ± 11.4). No significant differences in demographic variables (*e.g.*, age, height, BMI, lean body mass, body fat percentage, SBP, DBP, resting heart rate, thigh circumference, and waist circumference) were shown between the combined exercise group and the control group.

Changes in the physical fitness of the subjects after 12 wk of combined training

The changes in physical fitness variables for the subjects are shown in Table 3. The patients in the exercise group showed significant improvement in leg strength (42.5 ± 21.0 *vs* 62.6 ± 21.6, P = 0.009). The improvements in grip strength, YMCA step test, vertical jump, and sit and reach exhibited by the exercise group (grip strength, 25.8 ± 9.3 *vs* 28.0 ± 8.6; YMCA step test, 122.4 ± 16 *vs* 111.0 ± 13.9; vertical jump, 21.5 ± 10.1 *vs* 27.8 ± 11.2; and sit and reach 4.3 ± 8.9 *vs* 5.7 ± 8.8) were not significantly different from the control group (grip strength, 29.5 ± 10.0 *vs* 29.3 ± 8.2; YMCA step test, 120.3 ± 16.4 *vs* 119.6 ± 11.7; vertical jump, 21.5 ± 12.2 *vs* 24.3 ± 11.2; sit and reach 3.9 ± 8.5 *vs* 5.4 ± 8.1) (exercise *vs* control, P = 0.113, P = 0.084, P = 0.159 and P = 0.549 for grip strength, YMCA step test, vertical jump, and sit and reach, respectively).

Changes in segmental colon transit time among the subjects after 12 wk of combined exercise training

Table 4 shows colon transit times according to colon segments among the subjects after the 12-wk combined exercise program. Right colonic transit time (RCTT) (15.6 ± 15.2 *vs* 9.2 ± 11.9), left colonic transit time (LCTT) (19.7 ± 23.5 *vs* 10.4 ± 13.2), recto-sigmoid colonic transit time (RSCTT) (14.3 ± 16.7 *vs* 6.7 ± 7.9), and total colonic transit time (TCTT) (50.2 ± 38.1 *vs* 27.1 ± 28.0) decreased in the combined exercise group. In contrast, in the control group, only the RCTT (13.1 ± 10.4 *vs* 10.9 ± 18.7, P = 0.593) and LCTT (19.2 ± 19.0 *vs* 16.9 ± 19.8, P =0.489) were decreased, whereas the RSCTT (15.0 ± 14.4 *vs* 19.3 ± 30.3, P =

Table 4 Changes in segmental colon transit time among the subjects after 12 wk of combined exercise training

Variable	Combined exercise (n = 31)		Control (n = 21)		P
	Pre	Post	Pre	Post	
RCTT (h)	15.6 ± 15.2	9.2 ± 11.9	13.1 ± 10.4	10.9 ± 18.7	0.370
LCTT (h)	19.7 ± 23.5	10.4 ± 13.2[f]	19.2 ± 19.0	16.9 ± 19.8	0.207
RSCTT (h)	14.3 ± 16.7	6.7 ± 7.9	15.0 ± 14.4	19.3 ± 30.3	0.041[a]
TCTT (h)	50.2 ± 38.1	27.1 ± 28.0[h]	47.4 ± 34.6	47.3 ± 47.3	0.019[d]

The values are shown as the mean ± SD. The change in RSCTT reflects the difference in the time × group interaction between the exercise and control groups ([a]P = 0.041). The change in TCTT reflects the difference in the time × group interaction between the exercise and control groups ([d]P = 0.019). Significant differences between pre and post combined exercise group in LCTT ([f]P = 0.040). Significant differences between pre and post combined exercise group in TCTT ([h]P = 0.017). RCTT: Right colon transit time; LCTT: Left colon transit time; RSCTT: Recto-sigmoid colon transit time; TCTT: Total colon transit time.

0.412) increased. The items with significant differences between time points and groups were the RSCTT (P = 0.041) and TCTT (P = 0.019).

DISCUSSION

We analyze that the effect of combined exercise on CTT of mentally patients in a closed ward through a combined exercise program including resistance training with an elastic band and an aerobic exercise. Implementation of the combined exercise program resulted in a significant improvement in muscle strength and decreased colon transit time in the exercise group compared with that before the program.

In general, patients held in a closed ward lack PA because of long-term hospitalization and medical treatment. Therefore, various consequences, such as obesity, weakening of heart function, and depressive symptoms, manifest due to lethargy, weight increases, and muscle weakness. Problems such as lack of physical strength and symptoms of constipation in schizophrenic patients can be reduced by implementing the exercise program presented in this study as an alternative to medication.

The results of this study are meaningful, as they provide baseline physical fitness data among schizophrenic subjects and their data after a 12-wk combined exercise program aimed at schizophrenic patients. The results of the combined exercise program show that leg strength significantly improved.

Among the many studies examining the direct relationship between exercise and colon transit time between two groups of subjects, one study included one group that engaged in a sedentary lifestyle for one week and another group that participated in aerobic exercise 3 times in the same week. However, significant differences were not observed in colon transit time between the sedentary lifestyle group and the exercise group[24]. In addition, another study reported that 4 wk of aerobic exercise did not improve constipation[25]. However, in these previous studies, the exercise effect was observed over a relatively short period (1-4 wk). In this research, although the transit time of each colon segment decreased, it was not significantly reduced compared with that in the control group. The reduced colon transit time in the control group was attributed to the effect of balanced meals and a regular lifestyle during hospitalization. Although significant differences in the colon transit time of RCTT and LCTT were not observed, the RSCTT and TCTT were significantly reduced in the exercise group compared with those in the control group. The results of this research may be related to the relatively long-term (12 wk) observation period compared with that in previous research. Therefore, combined exercise can improve constipation by reducing the colon transit time, thus preventing colon cancer. Moreover, the colon transit time decreased despite general confounding variables, which were well controlled in this study because the patients in the exercise group and the control group were admitted to closed wards. Future research is required to examine the effects of exercise on colon transit time and constipation according to the dose of exercise, the exercise type, and exercise intensity in a greater number of subjects. This research showed that increased PA through long-term exercise (12 wk), in addition to eating habits, water intake, and dietary fiber intake, can reduce the colon transit time. Additionally, the results indicated that the risk of colon cancer can be reduced through exercise, which has an effect on functional constipation relief. This research is significant, as it provides basic evidence in support of prescribing exercise to specific patients who are admitted to a closed ward. In conclusion, among the patients who were admitted to a closed ward, participation in an exercise program improved leg strength and reduced colon transit times. Therefore, regular exercise is considered essential to improve physical health and reduce colon transit times among schizophrenia patients.

ARTICLE HIGHLIGHTS

Research background

The importance of exercise in the prevention of colorectal cancer has been well documented. However, an insufficient number of studies have examined the effects of the exercise or type of exercise on a colonic transit time (CTT). This study was performed to identify differences in CTT depending on the combined exercise. This study was designed to investigate the relationship between

combined exercise and colon transit time in mentally ill patients to elucidate how a combined exercise program could promote gastrointestinal (GI) motility.

Research motivation

The previous studies that have investigated the effects of increasing physical activity by participation in an exercise program and changes in CTT have largely involved healthy subjects with a normal lifestyle. However, no studies reported in the publicly available literature to date have reported on the effects of exercise on colonic function in mentally ill patients residing in a closed hospital ward. Therefore, we examined the effect of a combined exercise program on CTT among mentally ill patients who were admitted to a closed ward.

Research objectives

The research objective of this investigation was to examine the effects of physical activities and exercise on CTT in mentally ill patients in a closed ward and determine whether physical activity and exercise improve GI motility.

Research methods

Over a 6-mo period, 52 consecutive patients with mental illness were recruited from the Somang Hospital Psychiatry Unit. A combined exercise program was implemented 60 min per day, 3 d a week, for 12 wk. Fitness and CTT were measured twice before and twice after the exercise program. The CTT of patients in the two groups were compared for different clinical situations (combined exercise group vs control group).

Research results

The patients in the exercise group showed exercise-induced improvement in leg strength. Improvements in grip strength, YMCA step test, vertical jump, and sit and reach were also exhibited by the exercise group. Segmental colon transit times (RSCTT and TCTT) decreased in the combined exercise group. The results of this study are meaningful, as they provide physical fitness data for mentally ill patients at baseline and after a 12-wk combined exercise program aimed at this population.

Research conclusions

This research is significant, as it provides basic evidence in support of prescribing exercise to specific patients who are admitted to a closed ward. Among the patients who were admitted to a closed ward, participation in an exercise program improved leg strength and reduced CTT. Therefore, regular exercise is considered essential to improve physical health and reduce CTT among mentally ill patients.

Research perspectives

One of the most common signs of low GI motility is constipation. An important cause of low GI motility, and one of the easiest to correct, is physical inactivity. A long-term (12-wk) combined exercise program is sufficient to reap the potential benefits for GI motility and positively impact fitness and CTT to affect the overall health of mentally ill patients.

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CASE REPORT

Pancreaticoduodenectomy with combined superior mesenteric vein resection without reconstruction is possible: A case report and review of the literature

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Abstract

We report the case of a 56-year-old woman with pancreatic adenocarcinoma (PA) discovered during an episode of febrile jaundice. A computed tomography (CT) scan showed a mass in the head of the pancreas with circumferential infiltration of the superior mesenteric vein (SMV) and dilatation of the biliary and pancreatic ducts without metastases. The patient benefited from neoadjuvant chemotherapy (FOLFIRINOX) followed by radio-chemotherapy (45 Gy) and chemotherapy (LV5FU2). The revaluation CT revealed SMV thrombosis without portal vein (PV) thrombosis. There was no contact of the tumor with the PV. Pancreatoduodenectomy with combined resection of the SMV was performed with no reconstruction of this venous axis after confirmation of adequate PV, splenic, and left gastric venous flow and the absence of bowel ischemia. The pathological diagnosis was pT4N1R0 PA. There were no bowel angina issues during the follow-up period. At 15 mo after surgery, the patient died of metastatic recurrence.

Key words: Pancreatic ducts; Locally advanced; No reconstruction; Pancreatic adenocarcinoma; Superior mesenteric vein

Core tip: This case report showed that a short superior mesenteric vein resection could be achieved during pancreatoduodenectomy without venous reconstruction, when an appropriate small bowel venous outflow is ensured by inferior mesenteric vein.

Jouffret L, Guilbaud T, Turrini O, Delpero JR. Pancreaticoduodenectomy with combined superior mesenteric vein resection

without reconstruction is possible: A case report and review of the literature. *World J Clin Cases* 2018; 6(8): 214-218 Available from: URL: http://www.wjgnet.com/2307-8960/full/v6/i8/214.htm DOI: http://dx.doi.org/10.12998/wjcc.v6.i8.214

INTRODUCTION

Tumor resection is the only effective treatment option in patients with pancreatic adenocarcinoma (PA), and achieving macroscopic and microscopic complete resection (R0) is critical for long-term survival[1]. When the tumor involves either the superior mesenteric vein (SMV) or the portal vein (PV), SMV\PV resection is necessary at the time of pancreaticoduodenectomy (PD) to obtain his-tologically negative surgical margins[2,3]. Venous reconstruction is not usually a technical challenge, and end-to-end anastomosis is routinely performed with good 5-year patency rates[4]. In some case, involvement of first-order branches of the SMV increases the difficulty of venous reconstruction. We report the case of a patient presenting with PA with circumferential infiltration of the SMV and first-order branches in whom no venous reconstruction was performed.

CASE REPORT

A 56-year-old woman underwent abdominal computed tomography (CT) because of febrile jaundice. The CT scan revealed a mass (29 mm × 20 mm) in the head of the pancreas with circumferential infiltration of the SMV and dilatation of the biliary and pancreatic ducts. There was no metastasis.

An echo-endoscopy with retrograde catheterization was realized with stenting of the biliary duct. This exam affirmed the diagnosis of locally advanced PA. Neoadjuvant therapy was planned before surgical revaluation. The patient received neoadjuvant chemotherapy with 6 cycles of FOLFIRINOX followed by radiochemotherapy at 45 Gy over 5 wk associated with LV5-FU2 chemotherapy. The revaluation CT performed after neoadjuvant therapy revealed SMV thrombosis but no PV thrombosis and no contact with the tumor (Figure 1). There was no ar-gument for a tumor origin of thrombosis, it was not enhanced by contrast product. There were some collaterals vessels from SMV to the Inferior Mesenteric Venous (IMV). A multidisciplinary tumor board decided on surgical resection, and PD was planned.

On exploration, there was no evidence of metastasis or peritoneal nodularity. We first approached the superior mesenteric artery to be sure of the resectability of the tumor. The intraoperative frozen-section analysis result of the superior mesenteric artery margins was negative. After dissection, we found a point of tumor contact with the SMV. There was SMV thrombosis, but the PV and splenomesaraic confluence were tumorfree. PD combined with SMV resection was performed to obtain a negative surgical margin. We mechanically sectioned the distal extremity of the SMV and selectively ligated the ileum and jejunum veins under the tumor. No reconstruction of this venous axis was realized after confirming, by a clamping test, adequate PV, splenic, and left gastric venous flow and the absence of bowel ischemia. We performed a Child reconstruction with pancreatojejunostomy and external pancreatic duct stenting and drainage (Figure 2). Surgery lasted 10 hours. There was less bleeding, and no transfusion. We inked the tumor margins to differentiate areas of venous, arterial and posterior margin resection. The pathological diagnosis was PA with poor differentiation, lymph node metastasis (2N+/5), vascular and perineural invasion, and SMV wall infiltration, ypT3N1M0. All resection margins were tumor-free.

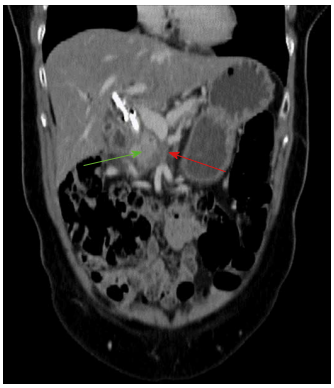

Figure 1 Initial computed tomography scan, axial section: Tumor of the pancreatic head (green arrow) with adjacent thrombus of the superior mesenteric vein (red arrow)

During the postoperative course, the patient developed isolated chylous ascites that disappeared spontaneously in a few weeks. Control CT after surgery showed an aspect of aspecific colitis with edema in the mesenteric structures of the colon. The patient was released from the hospital after fifteen days. A multidisciplinary tumor board decided there were no indications for adjuvant therapy because of clinical and nutritional state of patient. Subsequently, the patient experienced metastatic evolution with hepatic and pulmonary lesions at 6 mo. Unfortunately, the patient died of metastatic progression, 15 mo after the surgery, with no evidence of local recurrence.

DISCUSSION

In this case, no reconstruction after venous resection without the use of autologous or prosthetic grafts was feasible. As SMV thrombosis was observed before the surgery, venous drainage of the small bowel was achieved via the lower mesenteric and splenic veins, and reconstruction of the SMV was not necessary. PD with SMV resection and without reconstruction was performed in two another cases, as described by Hashimoto *et al*[5],

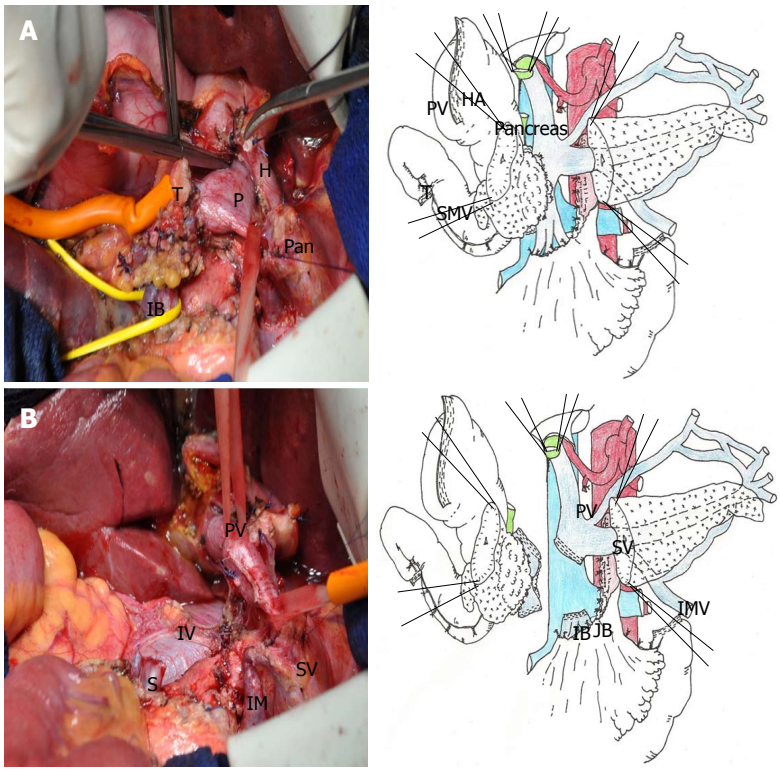

Figure 2 Operative view and illustration at the initial surgery. A: Involvement of pancreatic tumor (T) in the superior mesenteric vein (SMV) without involvement of the portal vein (PV); ileal branch of the SMV (IB); jejunal branch of the SMV (JB); hepatic artery (HA); stump of transected pancreas; B: No SMV reconstruction after tumor resection; venous return by inferior mesenteric vein (IMV) and splenic vein (SV); inferior vena cava (IVC).

after confirming adequate PV flow and no small intestine congestion, and by Tang *et al*[6], who performed the same operation with anastomosis between the splenic vein and PV without SMV reconstruction.

The poor prognosis of this condition is partly due to local invasion of the tumor into the tissues around the pancreas. In this case, PD combined with venous resection was performed to treat pancreatic head adenocarcino-ma with tumor-vein contact and/or invasion to obtain R0 resection. The rationale for radical surgical excision is based on the poorer survival rate in patients with incomplete resection (R1) (14-21 mo) than in patients with R0 resection (18-28 mo), as determined by the study with the largest series of cases performed in an specialized center[7-10]. The definition of R1 resection has not been standardized. Chang *et al*[11] showed that a tumor clearance greater than 1.5 mm in the retroperitoneal section was an independent predictor of long-term survival in a multivariate analysis in 365 patients. Delpero *et al*[12] demonstrated that the standardized and systematic inking of the retroperitoneal margin increased the rates of R1 resection; additionally, these researchers confirmed the importance of R0 resection with a significantly lower 2-year disease-free survival rate in patients with R1 resection (R1: 26.5% *vs* R0: 42%; $P = 0.02$). In patients with R1 resection, the site of microscopic involvement impacted survival, and patients with involvement of vascular margins had poorer survival than patients with involvement of posterior margins[13].

When a tumor is in contact with the PV, tumor invasion occurs in only 50%-65% of cases, and involvement of the PV is a factor of a poor prognosis[14,15]. However, venous resection could increase the rate of R0 resection and be justified to increase the clearance of the retroportal resection margins. Additionally, Turrini *et al*[16] showed better 3-year overall survival in patients with no venous involvement who underwent PV resection than in those who did not (42% *vs* 22%, $P = 0.04$).

Many studies have not found any significant differences in terms of postoperative morbidity and mortality after venous resection[17]. Long-term results after PV reconstruction have shown 1- and 5-year patency rates of 50%-80% and 17%, respectively. There have been some cases of early thrombosis after PV reconstruction, with no major complications. Stenosis of the PV reconstruction area has been shown to be associated with local recurrence[4].

In most cases requiring reconstruction, end-to-end anastomosis is used, but some authors have described the use of autologous venous grafts or prosthetic patches in cases with long resection distances, with similar postoperative outcomes. Therefore, some patients developed thrombosis earlier in their graft, leading to large-volume ascites requiring paracentesis[18].

The involvement of first-order branches of the SMV is a surgical challenge. In a report by Katz *et al*[19], segmental resection of one of the two first-order branches

of the SMV could be performed without reconstruction if the remaining branch is preserved and assures collateral mesenteric venous drainage.

Acute thrombosis of the PV and SMV can lead to mesenteric venous ischemia. Thus, the lack of feasible reconstruction has historically been a contraindication of pancreatic resection. Data from the trauma literature suggest that SMV ligation can be performed without dramatic consequences[20]. Therefore, in cases of chronic SMV obstruction by a tumor or previous thrombosis, the development of collateral flow through the inferior mesenteric and splenic veins allows ligation of the SMV.

In conclusion, before surgery, high-quality CT needs to be performed to identify chronic venous obstructions by tumor involvement or thrombosis, evaluate the development of venous collaterality, and determine the feasibility of resection with or without PV reconstruction.

ARTICLE HIGHLIGHTS

Case characteristics

A 56-year-old woman with a locally advanced pancreatic adenocarcinoma (PA).

Clinical diagnosis

Febrile Jaundicy.

Laboratory diagnosis

Ca 19-9 42, cholestasis decreased after biliary stenting.

Imaging diagnosis

Computed tomography scan revealed superior mesenteric vein (SMV) thrombosis but no PV thrombosis and no contact with head PA.

Pathological diagnosis

PA with poor differentiation, lymph node metastasis (2N+/5), vascular and perineural invasion, and SMV wall infiltration. All resection margins were tumor-free.

Treatment

Pancreaduodenectomy with SMV resection without reconstruction.

Related reports

No reconstruction of this venous axis was realized after confirming adequate portal veinous, splenic, and left gastric venous flow and the absence of bowel ischemia.

Term explanation

Development of collateral flow through the inferior mesenteric and splenic veins allows ligation of the superior mesenteric venous.

Experiences and lessons

This case report showed that a short SMV resection could be achieved during pancreatoduodenectomy without venous reconstruction, when an appropriate small bowel venous outflow is ensured by inferior mesenteric vein.

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CASE REPORT

Multimodal treatments of right gastroepiploic arterial leiomyosarcoma with hepatic metastasis: A case report and review of the literature

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Author contributions: Seo HI is the first author; Kim DI is the corresponding author of the manuscript; Chung Y and Choi CI analyzed and interpreted the patient data; Kim S reviewed radiologic findings; Park DY did the pathology reading of the slides; Yun S and Kim M were involved in drafting and revising the manuscript; all authors read and approved the final manuscript.

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Abstract

Leiomyosarcoma of an artery is very rare, and cases with hepatic metastasis are even rarer. We describe a case of a 70-year-old man who after follow up due to rectal cancer, presented with an intra-abdominal hypervascular mass and a hepatic mass. After surgical resection, it was diagnosed as a leiomyosarcoma of the right gastroepiploic artery with hepatic metastasis. Multiple metastases had recurred at the liver. He has survived more than 53 mo through multimodal treatments (three surgical resections, radiofrequency ablation, transarterial chemoembolization, chemotherapies, and targeted therapy). Multimodal treatments, including active surgical resection, may be

helpful in the treatment of aggressive diseases such as arterial leiomyosarcoma with metastasis.

Key words: Multimodal treatments; Intra-abdominal arterial leiomyosarcoma; Hepatic metastasis; Arterial leiomyosarcoma; Surgical resection

Core tip: An arterial leiomyosarcoma (aLMS) is a very rare and aggressive disease. The prognosis is also very poor. A 70-year-old man presented with an intra-abdominal aLMS with hepatic metastasis. He was treated with multimodal treatments that consisted of three surgeries, radiofrequency ablation, transarterial chemoembolization, and targeted therapy. He has survived for 53 mo after these treatments. Multimodal treatments could be helpful treating this kind of disease.

Seo HI, Kim DI, Chung Y, Choi CI, Kim M, Yun S, Kim S, Park DY. Multimodal treatments of right gastroepiploic arterial leiomyosarcoma with hepatic metastasis: A case report and review of the literature. *World J Clin Cases* 2018; 6(8): 219-223 Available from: URL: http://www.wjgnet.com/2307-8960/full/v6/i8/219.htm DOI: http://dx.doi.org/10.12998/wjcc.v6.i8.219

INTRODUCTION

The leiomyosarcoma (LMS) is very rare malignant tumor. They usually originate in the smooth muscle of the soft tissues and uterus[1]. About 2% of LMS cases occur in the smooth muscle of the vessel wall and 60% occur in the inferior vena cava. The occurrence of LMS involving the veins is about five times higher than that of the arteries[1]. The most common site of arterial LMS (aLMS) is the peripheral artery, and the intra-abdominal artery is a rare location for aLMS to occur[1]. To the best of our knowledge, this is the first presentation of intra-abdominal aLMS with distant single liver metastasis. We report the clinical course of aLMS that originated from the right gastroepiploic artery with hepatic metastasis during multimodal treatments [three surgical resections, radiofrequency ablation (RFA), transarterial chemoembolization (TACE), chemotherapy, and targeted therapy] and review the literature regarding aLMS.

CASE REPORT

This case involves a 70-year-old man who had a previous operation history due to renal cell carcinoma and rectal cancer (pT2N0M0, stage IIA), ten years and six months ago. Abdominal computed tomography (CT) and magnetic resonance imaging (MRI) performed six months after the low anterior resection revealed a new 47 mm hypodense hepatic mass and a 23 mm hypervascular mass at the great curvature side of stomach (Figure 1A and 1B). It was highly suspected to be a malignant gastrointestinal stromal tumor (GIST) with hepatic metastasis. Fluorine-18 fluorodeoxyglucose positron emission tomography/computed tomography (FDG PET/CT) demonstrated a hypermetabolic low-density lesion in S8 of the liver and the greater curvature of the stomach with a maximum standardized uptake value (SUVmax) of 3.4 and 2.3, respectively (Figure 1C). For confirmation of the diagnosis, we planned to perform an ultrasonography-guided core needle biopsy. The core needle biopsy specimen of the liver mass showed malignant spindle cell with increased mitosis (7/10 HPFs). Immunohistochemistry results were positive for desmin and smooth muscle actin (SMA) and negative for CD34, c-kit, DOG-1, S-100, and HMB45. These findings suggest that aLMS was the proper diagnosis, not GIST. The mass in the greater curvature of the stomach showed high vascularity upon endoscopic ultrasonography. Therefore, a fine needle aspiration biopsy was not performed because of a bleeding risk. Laparotomy was performed with a diagnosis of the omental GIST and primary hepatic LMS. The omental mass resection and S8 segmentectomy were performed. The omental mass originated from the right gastroepiploic artery on surgical and microscopic field. This mass was a 3.0 cm × 2.7 cm sized aLMS (Figure 2A and 2B). Histopathology showed moderate cellular atypia, high mitotic rate (10/10 HPFs), and 2/3 histologic grade according to the FNCLCC grading system. Ki-67 proliferation index was 4.1% (Figure 2C). Immunohistochemistry results were positive for CD34, CD31, desmin, and SMA and negative for c-kit, DOG-1, and S-100. The liver mass was a 5.0 cm × 3.0 cm × 1.5 cm sized metastatic aLMS with a clear resection margin (free margin: 0.3 cm). Ki-67 proliferation index was 9.3%. The patient was discharged on the nine days after the operation without any complications. It was planned that four cycles of adriamycin monochemotherapy would be administered as an adjuvant treatment. However, treatment was stopped after the third treatment because of neutropenic fever.

Abdominal CT was performed every three months after the operation to check for recurrence. At fourteen months after the first operation, CT and MRI revealed a 2.7 cm, a 5 mm, and an 8 mm sized metastatic masses on of the liver. No extrahepatic metastasis was noted on FDG PET/CT. Right anterior sectionectomy was performed fifteen months after the first operation. There were a 3.0 cm × 2.7 cm and a 1.0 cm × 1.0 cm sized metastatic vascular LMSs. Histopathology and immunohistochemistry showed a high mitotic rate (22/10 HPFs) and a Ki-67 proliferation index of 4.1%. The resection margin was very close to the mass (< 1 mm). Ifosfamide monochemotherapy was administered after the surgery for four cycles.

At eight months after the second operation, CT, MRI and FDG PET/CT revealed a 1.6 cm and a 1.4 cm sized seeding metastatic nodules on the diaphragm and the liver. Diaphragm partial resection and intra-operative

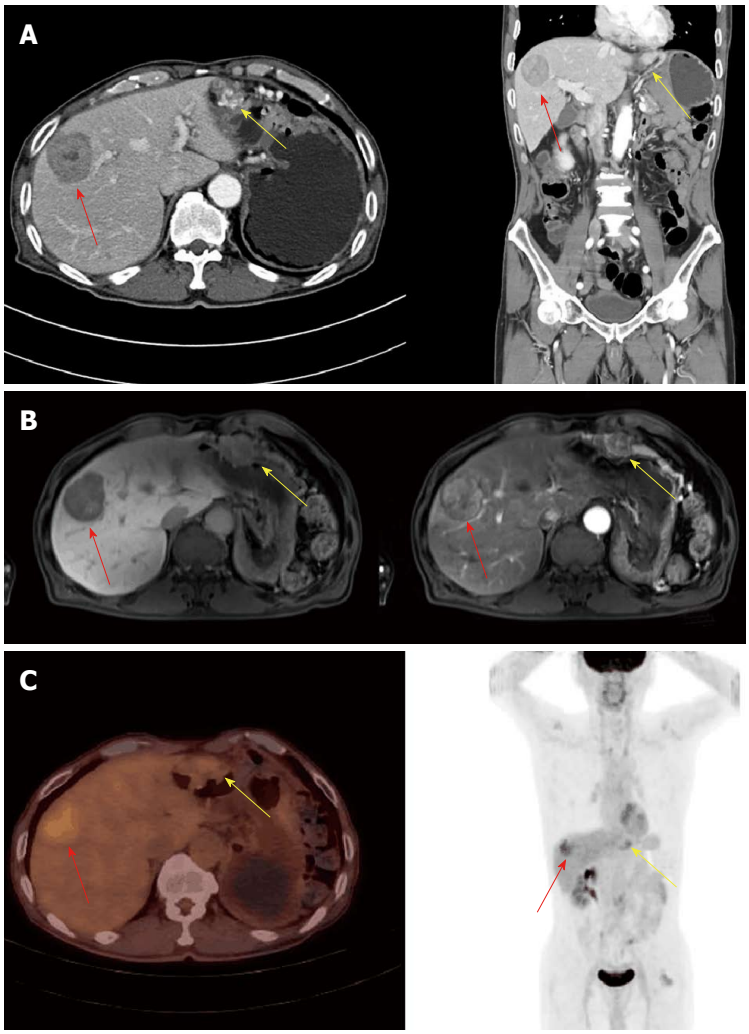

Figure 1 Diagnosis imaging of the patient. A: Computed tomography showed a 43 mm hypodense mass at S8 of the liver (red arrow) and a 23 mm sized hypervacular mass at the great curvature side of stomach (yellow arrow). B: Magnetic resonance imaging showed a well-defined encapsulated lesion (red arrow) in S8 of the liver, which showed a strong enhancement during the arterial dominant phase, with wash out during the delayed phase. The mass (red arrow) in the greater curvature of the stomach was accompanied by engorgement of the gastroepiploic vein. C: Fluorine-18 fluorodeoxyglucose positron emission tomography/computed tomography images showed a hyper-vascular mass at the great curvature side of stomach (yellow arrow) and a hypervascular metastatic mass at S8 of liver (red arrow).

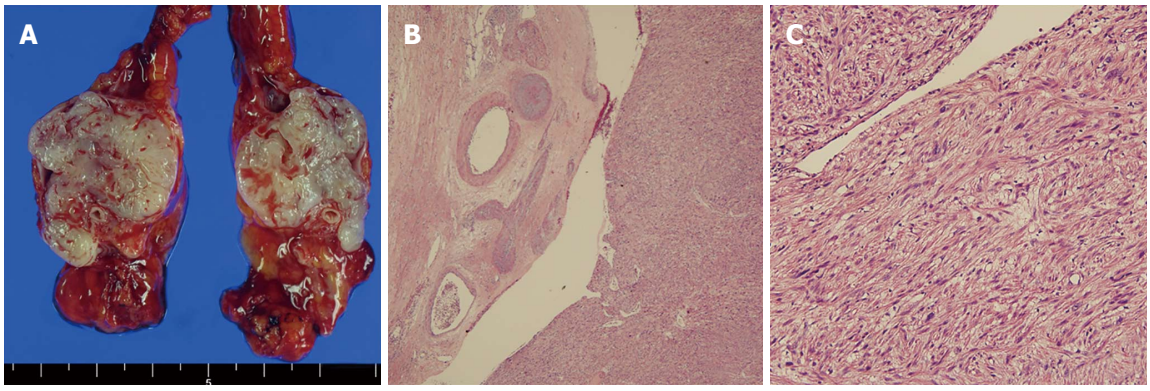

Figure 2 The gross find of arterial leiomyosarcoma and pathological diagnosis. The omental mass and A: Gross finding of arterial leiomyosarcoma. A white colored expending nodular mass was present on soft tissue. A medium sized artery was present on adjacent connective tissue. B: Tumor consisted of spindle cells and adjacent to medium sized vessels (H and E staining, × 40). C: Tumor cells showed spindle shaped nucleus with rounded end and eosinophilic cytoplasm (H and E staining, × 200). They formed fascicular pattern and frequently made stage horn shaped vascular spaces.

Table 1 Review of intra-abdominal arterial leiomyosarcoma literature

Ref.	Sex	Age (yr)	Site	Symptom	Treatment	Metastasis	Follow-up
Hopkins[2] (1968)	M	55	Common iliac	Claudication	Surgery	-	Died after 3 wk
Birkenstock *et al*[3] (1976)	M	55	Common iliac	Pain, palpable mass	Surgery	-	No evidence of disease
Stringer[4] (1977)	M	49	IMA	Pain, palpable mass	Surgery, RTx, CTx	Lung	Died after 7 yr
Gutman *et al*[5] (1986)	F	55	Common iliac	Claudication	Surgery	-	No evidence of disease
Delin *et al*[6] (1990)	F	72	Common iliac	Claudication, pain	Surgery	-	Died after 7 mo
Gill *et al*[7] (2000)	F	76	Renal	Pain	Surgery	-	Follow up for 9 mo
Rohde *et al*[8] (2001)	F	51	Splenic	Pain, weight loss	Surgery + CTx (adriamycin + ifosphamide)	-	Follow up for 1 yr
Blansfield *et al*[9] (2003)	F	42	Common iliac	Pain	Surgery	-	No evidence of disease
Current case	M	70	Right gastroepiploic	None	Multimodal	Liver	follow up for 36 mo

IMA: inferior mesenteric artery; RTx: radiotherapy; CTx: chemotherapy.

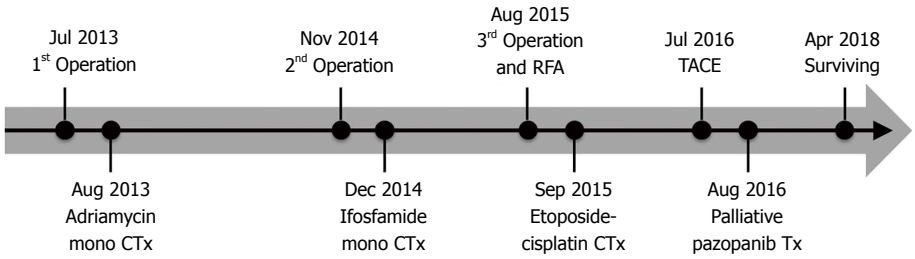

Figure 3 Timeline of multimodal treatments. CTx: chemotherapy; RFA: radiofrequency ablation; TACE: transarterial chemoembolization

RFA was performed nine months after the second operation (twenty-four months after the first operation). The diaphragm mass was also diagnosed as a metastatic vascular LMS. Etoposide-cisplatin chemotherapy was administered after the surgery for six cycles.

At eleven months after the third operation, there were multiple recurrences in the remnant liver as shown by MRI and TACE (2 mL lipiodol and 10 mg adriamycin) Palliative pazopanib was also started. The patient has been followed for twenty-eight months after the third operation (fifty-three months after the first operation). He is surviving with stable hepatic metastasis controlled by chemotherapy. Treatment modalities are summarized in Figure 3.

DISCUSSION

Over half of aLMS cases originate from the pulmonary artery, followed by the extra-abdominal peripheral artery. Including this case, only nine cases of aLMS originating from the intra-abdominal arteries, excluding the aorta, have been reported (Table 1)[2-9].

Compared to the other origin of LMS, the case of vascular LMS has a worse prognosis. In the case of LMS with metastasis, metastatic vascular LMS shows similar results as metastatic LMS of other origins[1]. However, the prognosis is not well-known owing to the low number of cases in aLMS. It is presumed that aLMS may be more aggressive than LMS, and hence, the prognosis is expected to be about the same or worse[1]. Because aLMS is more aggressive than LMS, and directly seed to artery, it has a higher possibility of metastasis[10].

Clinical signs of aLMS are diverse depending on the area of origin and most of them are due to the mass effect[9,10]. In this case, the 3 cm primary mass was located in the intra-abdominal area. The patient had no symptoms owing to aLMS. Although, the size of the primary cancer of the right gastroepiploic artery was 3 cm, which was small, it was accompanied by a distant metastasis at the time of diagnosis.

For diagnostic confirmation, a biopsy is needed, and radiological assisted core needle biopsy is preferred over open biopsy because there is a low risk of complications[10]. However, like this case, if the mass is located in the intraperitoneal region and shows hypervascularity, bleeding could occur after the core needle biopsy. Moreover, bleeding control could be difficult in this location. PET-CT showed SUVmax values of 3.4 and 2.3 each, which had relatively low uptake at first, and uptake was not noted at the lesion recurrence. More precise imaging studies are needed to overcome this limitation.

There are reports of treating LMS cases with surgical

resection, radiotherapy, and chemotherapy[1,4,6,9]. Chemotherapy or chemoembolization has been the main treatment of LMS with hepatic metastasis[10]. Recently, RFA also shows a good result for metastatic LMS[10]. However, recently, just like in other cases of metastatic cancer, liver resection shows better results[11]. If a resection of metastasis is possible, surgical treatment and additional treatment including chemotherapy can lead to a good response.

Although aLMS showed aggressive clinical features, multimodal treatment (resection, chemotherapy, RFA, chemoembolization, and targeted therapy) might be helpful to manage this kind of disease.

ARTICLE HIGHLIGHTS

Case characteristics

A 70-year-old man presented with an intra-abdominal mass and a hepatic mass during a follow visit for rectal cancer surgery.

Clinical diagnosis

After CT and magnetic resonance imaging (MRI), it was diagnosed as a malignant gastrointestinal stromal tumor (GIST) with hepatic metastasis.

Differential diagnosis

After the core needle biopsy of the liver, it was diagnosed as a leiomyosarcoma (LMS). Before the surgery, these were omental GIST and hepatic LMS.

Imaging diagnosis

At first, it was diagnosed a omental GIST and hepatic metastasis in CT and MRI.

Pathological diagnosis

The surgical specimen diagnosed as an aLMS with hepatic metastasis.

Treatment

Multimodal treatments were done (three surgeries, chemotherapy, transarterial chemoembolization, radiofrequency, and targeted therapy).

Related reports

There were only nine reports about intra-abdominal arterial leiomyosarcoma (aLMS). This is the first report of intra-abdominal aLMS with hepatic metastasis.

Term explanation

aLMS is a very rare and aggressive disease. The prognosis is very poor. There were few reports of this disease. So the treatment is also not established.

Experiences and lessons

Active treatments using multiple modalities may be helpful for these kinds of patients.

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CASE REPORT

Must Peutz-Jeghers syndrome patients have the *LKB1/STK11* gene mutation? A case report and review of the literature

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Author contributions: Gu GL designed the research; Duan FX, Gu GL, Yang HR and Yu PF prepared the samples for sequencing; Duan FX and Zhang Z conducted the sequencing; Gu GL and Duan FX collected and analyzed the data; Duan FX wrote the manuscript; Gu GL revised the manuscript.

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Abstract

Peutz-Jeghers syndrome (PJS) is an autosomal dominant inherited disease, which is characterized by mucocutaneous pigmentation and multiple gastrointestinal hamartoma polyps. The germline mutation of *LKB1/STK11* gene on chromosome 19p13.3 is considered to be the hereditary cause of PJS. However, must a patient with PJS have the *LKB1/STK11* gene mutation? We here report a case of a male patient who had typical manifestations of PJS and a definite family history, but did not have *LKB1/STK11* gene mutation. By means of high-throughput sequencing technology, only mutations in *APC* gene (c.6662T > C: p.Met2221Thr) and *MSH6* gene (c.3488A > T: p.Glu1163Val) were detected. The missense mutations in *APC* and *MSH6* gene may lead to abnormalities in structure and function of their expression products, and may result in the occurrence of PJS. This study suggests that some other genetic disorders may cause PJS besides *LKB1/STK11* gene mutation.

Key words: Peutz-Jeghers syndrome; Gastrointestinal polyps; High-throughput sequencing; *LKB1/STK11*; *APC*; *MSH6*; Hamartoma

Core tip: The germline mutation of *LKB1/STK11* gene on chromosome 19p13.3 is considered to be the hereditary cause of Peutz-Jeghers syndrome (PJS). We report a male PJS patient, who had typical manifestations and a definite family history, and but did not have *LKB1/STK11* gene mutation. By means of high-throughput sequencing technology, only mutations in *APC* gene (c.6662T > C: p.Met2221Thr) and *MSH6* gene (c.3488A > T: p.Glu1163Val) were detected in this case. This study suggests that some other genetic disorders may cause PJS besides *LKB1/STK11* gene mutation.

Duan FX, Gu GL, Yang HR, Yu PF, Zhang Z. Must Peutz-Jeghers syndrome patients have the *LKB1/STK11* gene mutation? A case report and review of the literature. *World J Clin Cases* 2018; 6(8): 224-232 Available from: URL: http://www.wjgnet.com/2307-8960/full/v6/i8/224.htm DOI: http://dx.doi.org/10.12998/wjcc.v6.i8.224

INTRODUCTION

Peutz-Jeghers syndrome (PJS) is an autosomal dominant inherited disease characterized by mucocutaneous pigmentation and multiple gastrointestinal hamartoma polyps[1]. PJS is a very rare disease, with an incidence of about 1/25000[2]. It has prominent clinical manifestations, and serious clinical hazards since the gastrointestinal hamartoma polyps of PJS can lead to severe complications, such as gastrointestinal bleeding, bowel obstruction, intussusception and cancerization. PJS hamartoma polyps are mainly distributed in the small intestine (especially the proximal jejunum), so PJS has a persistent disease course and concealing lesion site[3-5]. This may cause mental stress and economic burden on the patients and their families. Therefore, it is necessary for us to study PJS.

The germline mutation of *LKB1/STK11* gene on chromosome 19p13.3 is considered as the hereditary cause of PJS[6,7]. As a tumor suppressor gene, *LKB1/STK11* contains 9 exons and 11 introns. At present, the detected mutation rate of *LKB1/STK11* gene is 80%-94% by direct sequencing technology and multiplex ligation-dependent probe amplification (MLPA)[8-10]. This may suggest that the genetic etiology of PJS is heterogeneous[11-13]. Are there any other genetic causes for PJS besides *LKB1/STK11* gene? Here, we report a case of male patient with PJS who had missense mutations of *APC* gene and *MSH6* gene, but without mutations of *LKB1/STK11* gene.

CASE REPORT

Patient information

A 32-year-old male patient with PJS was hospitalized for endoscopic polypectomy in January 19, 2015. He had black spots on lips, buccal mucosa and fingertips since the age of 7 (Figure 1), and intermittent abdominal pain and black stool since the age of 25. He was diagnosed with PJS and underwent multiple endoscopic polypectomies to remove hamartoma polyps in the gastrointestinal tract *via* gastroscopy, enteroscopy and colonoscopy. His father was also diagnosed with PJS. This patient was in full compliance with the clinical diagnostic criteria for PJS issued by the WHO[14]: Patients without a family history can be diagnosed with PJS if they have three or more histopathologically confirmed PJS polyps, or characteristic, notable, mucocutaneous pigmentation with any amounts of PJS polyps. Patients with a family history can be diagnosed with PJS if they have any amounts of PJS polyps, or characteristic, notable, mucocutaneous pigmentation.

The abdominal and pelvic CT scan showed that there were many different sizes of polyps distributed in the descending duodenum and group 3rd-group 6th of the small intestine, and the local intestinal canal was torsional (Figure 2). Transanal double-balloon electronic enteroscopy revealed that there were multiple long pedicle, subpedicle or sessile polyps sized 0.5-1.2 cm in the colorectum, especially the descending colon, sigmoid colon and rectum. In addition, there were many huge lobulated polyps at a distance of 40 cm from the ileocecal valve that caused intestinal tract blockage. The patient underwent laparoscopic exploration, intussusception reduction, endoscopic small intestinal polyp cauterization, and small intestinal multiple hamartoma resection in January 25, 2015 (Figure 3). Postoperative pathological report showed PJS hamartoma polyps without canceration (Figures 4 and 5).

DNA extraction, quantification and quality control

We used paraffin tissue DNA extraction kit to extract DNA from the polyp tissue according to the instructions of the kit (QIAamp Tissue DNA FFPE Tissue Kit, QIAGEN, QIAGEN Strasse 1407124 Hilden, QIAGEN). Preliminary screening of the sample DNA was carried out using the peak map, and purity test was done through the ultraviolet spectrophotometer (Nanodrop 2000, Thermo Scientific, Wilmington, DE, United States). Accurate quantitative analysis of complete DNA fragments in the sample DNA was performed using the fluorescence quantitative instrument (The Qubit 2 Fluorometer, Thermo Scientific, Wilmington, DE, United States). The results showed that the concentration of the sample DNA (A260/A280 2.01) was 1314 ng/μL, and the concentration of the complete DNA fragments was 160 ng/μL, which was higher than the requirement of 10 ng/μL.

cDNA libraries construction and sequencing

We constructed cDNA libraries with Ion AmpliSeq Library Kit 2 according to the instructions of the manufacturer (Life 5791, Van Allen, Way Carlsbad. CA, United States). Ion Ampliseq Custom (IAD72340_182_pool 1, IAD72340_182_pool 2) (Life Technologies) was used to prepare a gene panel consisting of a primer pool for the entire coding region and exon-intron boundaries in 14 genes which often mutated in hereditary colorectal

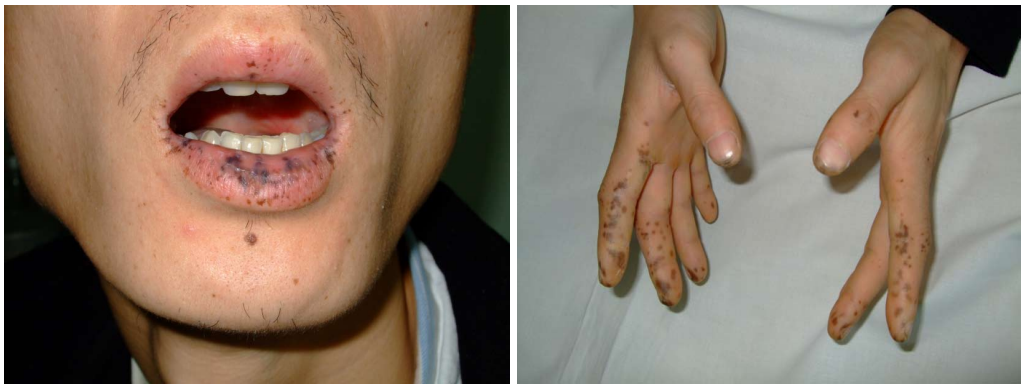

Figure 1 Mucocutaneous pigmentation of the patient.

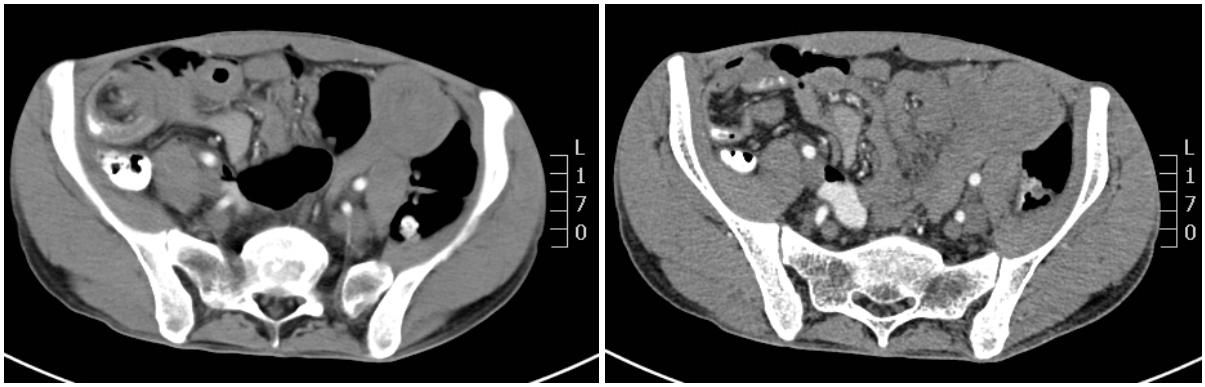

Figure 2 Abdominal and pelvic CT scan. It showed that many polyps are distributed in the descending duodenum and the small intestine, and the local intestinal canal was torsional.

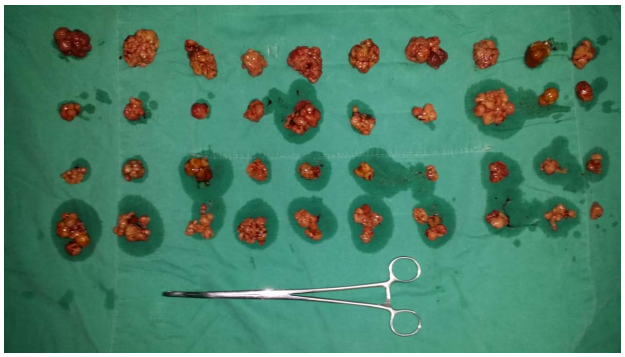

Figure 3 Hamartoma polyps removed from the patient's gastrointestinal tract.

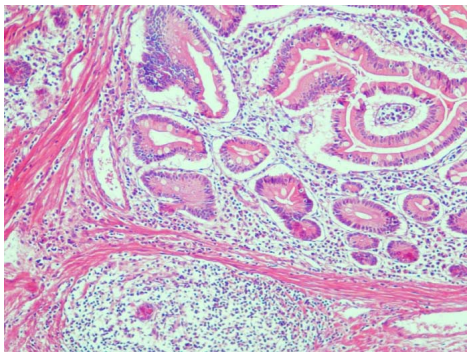

Figure 4 Postoperative pathological reports showed Peutz-Jeghers syndrome hamartoma polyps (HE, original magnification × 200).

cancer (*APC, AXIN2, BMPR1A, EPCAM, MLH1, MLH3, MSH2, MSH6, MUTYH, PMS1, PMS2, PTEN, SMAD4, LKB1/STK11*). After amplification, the libraries were purified using paramagnetic particle method (AMPure XP reagent, Beckman, United States). The fluorescence quantitative PCR instrument (ViiA 7 Dx, Life, Technologies Holdings Pte Ltd Block, Singapore) was used to detect the quantitative index of the libraries. Then the template was dealt with Ion OneTouch 2 template system (Life Technologies, Carlsbad, CA, United States) for preparation (Ion OneTouch2) and enrichment (Ion OneTouch ES). Finally, the high-throughput sequencing was performed on Ion PGM (Life, Technologies Holdings Pte Ltd Block, Singapore) using Ion 316 CHIP (Life Technologies Corp, Carlsbad, CA, United States).

Statistical analysis

The quality control parameters of the sequencing data were as follows: percentage reads on target > 85%, uniformity of base coverage > 85%, average base coverage depth: 200 ×-500 ×. The data were analyzed by Torrent Suite software (Life Technologies, v5.0.4) and compared with database of hg19 human

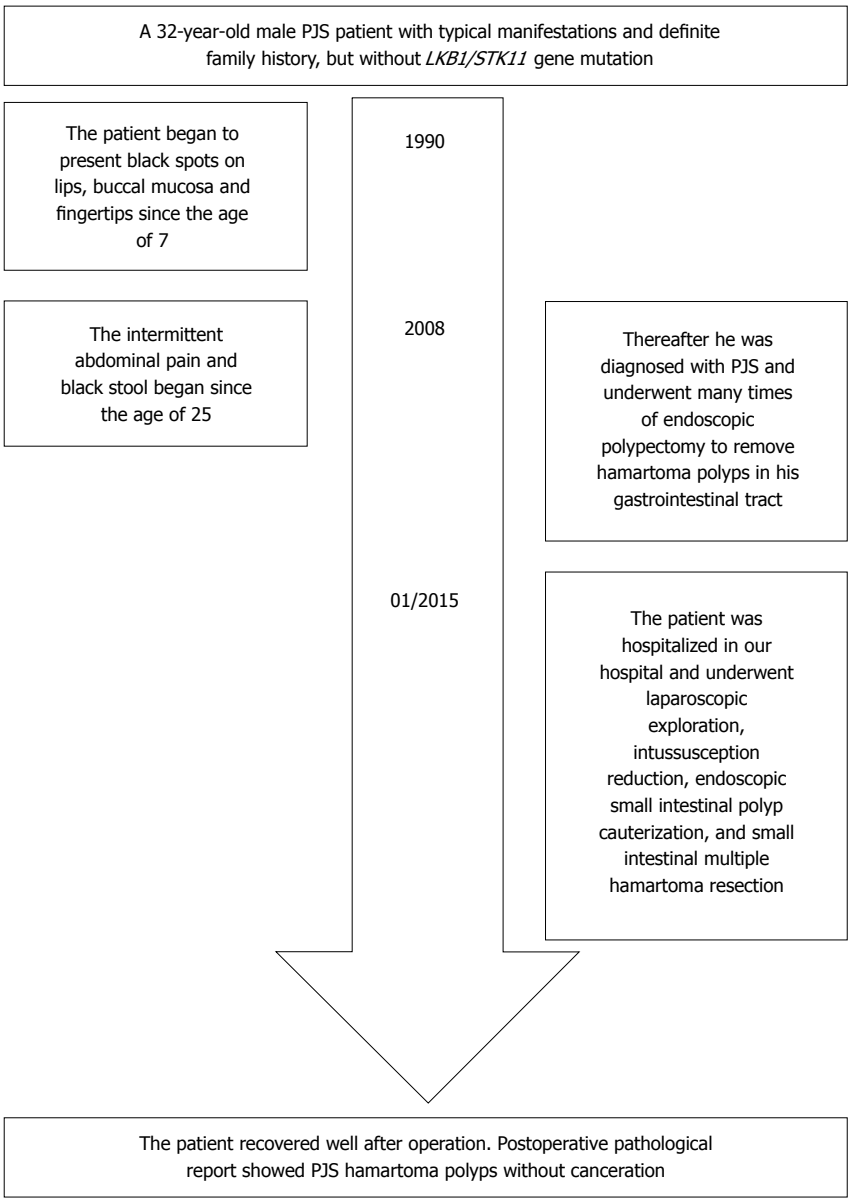

Figure 5 Timeline of the patient. PJS: Peutz-Jeghers syndrome.

reference genome. The detected gene mutations were annotated by Ion Reporter software (https://ionreporter.lifetechnologies.com/ir/secure/home.html) and ANNOVAR package (http://wannovar.wglab.org/).

Preliminary screening of mutation sites for verification was carried out according to the mutation frequency. Allele frequency database of the herd was based on the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/), 1000 Genomes Project (http://ftp.ncbi.nih.gov/) and the genome aggregation database (gnomAD, http://gnomad.broadinstitute.org/). According to the HGMD (version 2017.03, http://www.hgmd.cf.ac.uk/ac/index.php), mutation sites of minor allele frequency < 0.01 and the suspected or pathogenetic sites with frequency of 0.01-0.05 were retained for verification.

The Sanger sequencing technology was applied to candidate sites verification. Protein function was predicted with the software Polymorphism Phenotyping V2 (PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/index.shtml), MutationTaster (http://www.mutationtaster.org/), functional analysis was performed using the Hidden Markov Models (FATHMM, http://fathmm.biocompute.org.uk/index.html) and Mendelian Clinically Applicable Pathogenicity (M-CAP, http://bejerano.stanford.edu/MCAP/). GERP++(http://mendel.stanford.edu/SidowLab/downloads/gerp/index.html) and PhyloP (http://compgen.bscb.cornell.edu/phast) were used to forecast the conservatism of the impaired amino-acid residues.

Quality control data

This sequencing could read 28269827 bases. The base number of Q20 was 24754457, accounting for 87.56%. Percentage reads on target was 92.12%. Average base coverage depth was 286 × (coverage ⩾ 20 ×

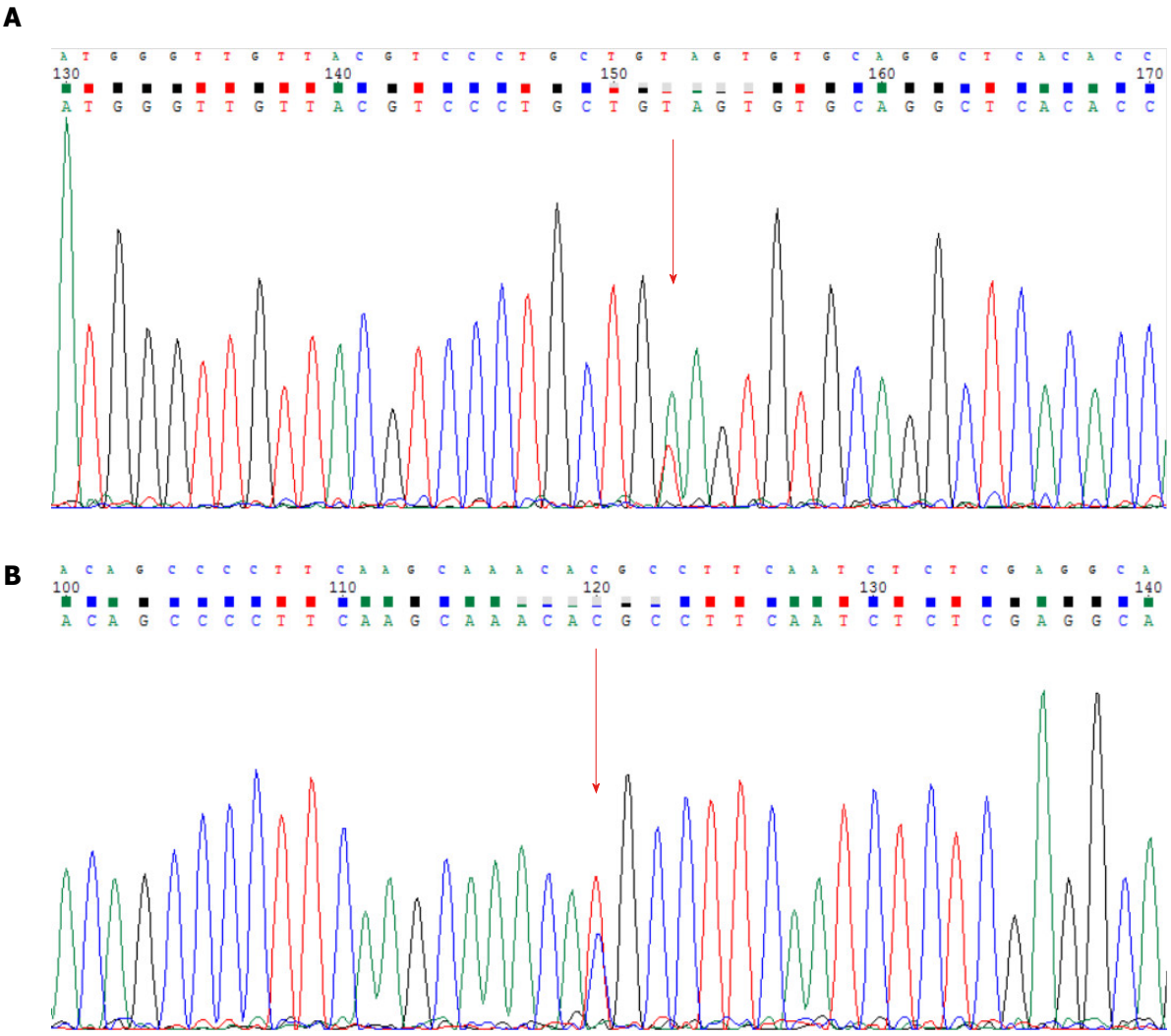

Figure 6 Results of the Sanger sequencing. Mutation sites are marked with arrows. A: *MSH6*: c.3488A>T; B: *APC*: c.6662T>C.

94.39%, coverage ⩾ 100 × 77.38%, coverage ⩾ 500 × 12.75%). Uniformity of base coverage was 92.12%.

Sequencing results

No mutation of *LKB1/STK11* gene was found in this patient, but he carries missense mutations in exon 6 of *MSH6* gene and exon 16 of *APC* gene (*MSH6*: NM_000179.2: exon6: c.3488A>T: p.Glu1163Val; *APC*: NM_000038.5: exon16: c.6662T>C: p.Met2221Thr) (Figure 6). The Sanger sequencing results proved the existence of these mutations (primers: *APC*: forward primer: CCAGGGGAGAAAAGTACATTGGA, reverse primer: ACTTGTACTTGAGGAGCTATTTCG; *MSH6*: forward primer: ACAGAACCAACGTACATGTGA, reverse primer: TTCTGTCTGAGGCACCAAGTC).

Prediction of protein function and amino-acid residue conservatism

The changes in the structure and function of *APC* protein caused by gene mutations were analyzed and determined as benign by Polyphen-2, as disease caused by MutationTaster, and as damage by FATHMM and by M-CAP. Evolutionary conservative analysis software of GERP++ and phyloP showed that the impaired amino-acid residue was conserved in different species, suggesting that the mutation may be pathogenic.

The structure and function changes of the mutated MSH6 protein were analyzed and defined as possible damage or benign by Polyphen-2, as disease caused by MutationTaster and as damage by FATHMM. Evolutionary conservative analysis software of GERP++ and phyloP indicated that the mutant amino-acid residue was most evolutionarily conserved and was likely pathogenic (Tables 1 and 2).

DISCUSSION

In this study, the patient had a clear family history and typical clinical manifestations of PJS, and histologic examination of his polyps proved to be hamartoma. However, we only detected the missense mutations in the *APC* gene and *MSH6* gene from his polyp tissue by high-throughput sequencing and Sanger sequencing technology, but no mutation in the *LKB1/STK11* gene.

Table 1 Prediction of protein function using different softwares

Gene	Polyphen-2_HDIV		Polyphen-2_HVAR		MutationTaster		FATHMM		M-CAP	
	Score	Prediction	Score	Prediction	Score	Prediction	Score	Prediction	Score	Prediction
MSH6	0.670	Possibly damaging	0.411	Benign	1	Disease causing	-2.12	Damaging	-	-
APC	0.156	Benign	0.026	Benign	0.737	Disease causing	-2.47	Damaging	0.046	Damaging

Table 2 Prediction of amino-acid residue conservatism

Gene	Exon	Protein	Coding	GERP++		PhyloP	
				Score	Prediction	Score	Prediction
MSH6	6	p.Glu1163Val	c.3488A > T	5.23	Conserved	8.923	Conserved
APC	16	p.Met2221Thr	c.6662T > C	6.02	Conserved	3.925	Conserved

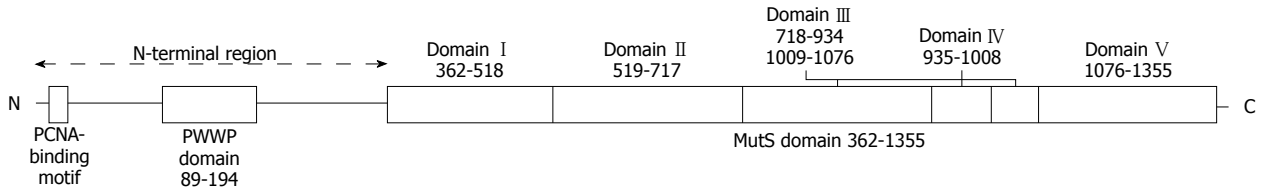

Figure 7 Domain organization of human MSH6[26]. It mainly consists of PCNA-binding motif, PWWP domain and the MutS domain.

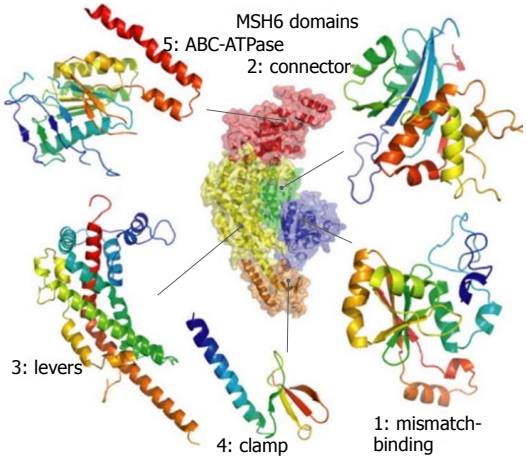

Figure 8 The domain structure of *MSH6*. Figures were generated with PyMOL[29].

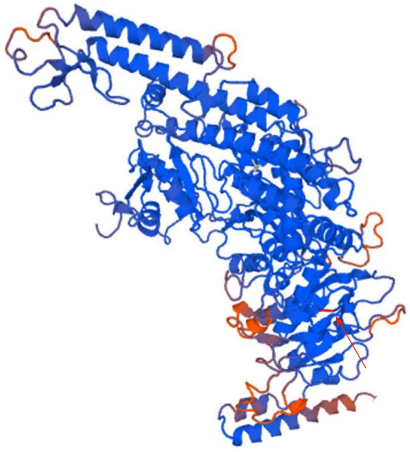

Figure 9 Structure of the mutant *MSH6* (p.Glu1163Val). Arrow indicates the position of the mutation. The figure was generated with Swiss-Model online software.

The mutation detected in *APC* gene has not been reported previously, and the MAF was 8.3e-6 according to the ExAC database. The mutation detected in *MSH6* gene has been reported in some sequencings[15-17], the MAF was 0.0012 according to the ExAC database as well as 0.0028 from 1000G database.

The DNA mismatch repair proteins (MMR), including MLH1, MSH2, MSH3, MSH6, PMS1 and PMS2, could remain gene stability by means of discovering and repairing mismatched bases and insertion/deletion loops improperly incorporated during DNA synthesis[18-21]. Expression deletion of MMR protein can be found in about 15%-25% sporadic tumors of various tissues[22]. The *MSH6* gene is located on chromosome 2p16.3, which contains 24 000 base pairs and 10 exons. The MSH6 protein was first reported as G/T mismatch repair binding protein (GTBP) in 1995, it constitutes MutSα complex with MSH2[23-25]. The main domains of MSH6 protein from N-terminal to C-terminal included (Figure 7): PCNA-binding motif, PWWP domain and the MutS domain[26-28]. The MutS domain is composed of five parts: (1) Mismatch binding domain, including the 362-518th amino acids; (2) connector domain, including the 519-717th amino acids; (3) levers domain, including the 718-934th and 1009-1075th amino acids; (4) clamp domain, including the 935-1008th amino acids; (5) ABC-ATPase domain, including the 1076-1355th amino acids[29] (Figure 8). The gene mutation (c.3488A>T) detected in our patient led the 1163rd amino acid of glutamate to become valine in the MSH6 protein (Figure 9). Changing an acidic amino-acid into a non-polar hydrophobic amino-acid may cause the ABC-ATPase

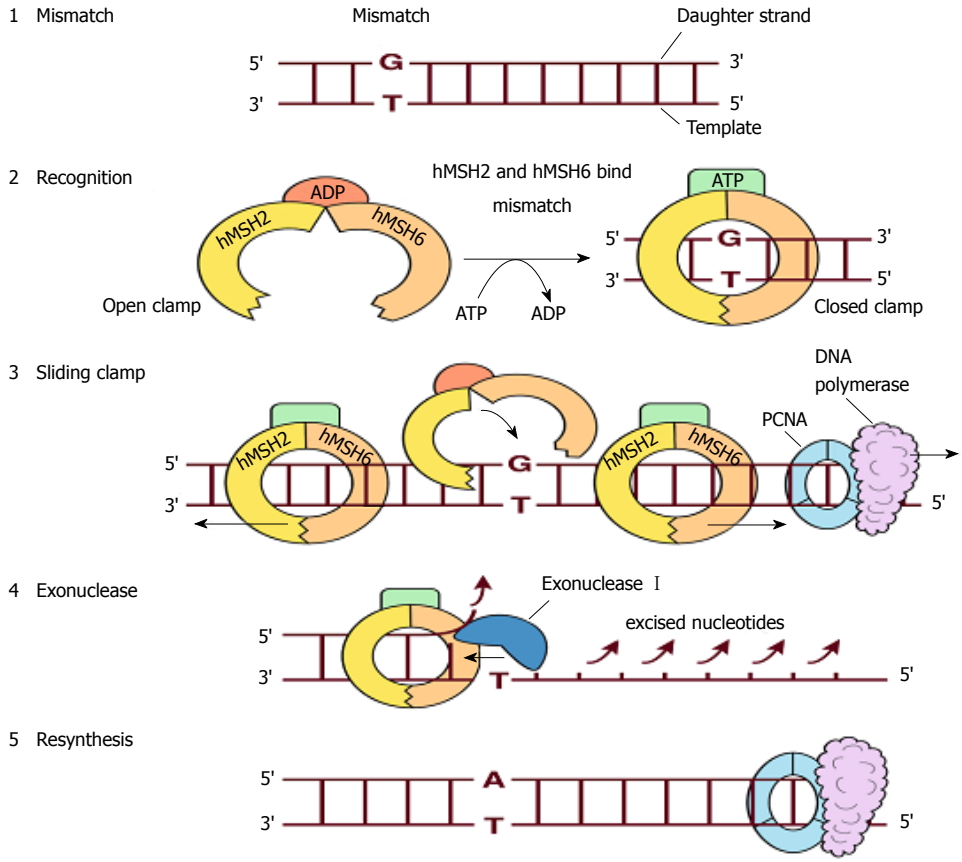

Figure 10 Repairing single-base mismatch in S phase by MutSα[30]. 1: A mismatch appears in the daughter strand; 2: Upon encountering the mispair, the MutSα, which consists of hMSH2, hMSH6 and ADP, switches to a closed, sliding clamp along the DNA. This process is accompanied by exchanging of ATP for ADP; 3: Multiple MutSα clamps may be recruited to the mismatch. Moving in the 5' > 3' direction, the MutSα will meet and displace the PCNA-DNA polymerase complex in DNA synthesis; 4: Exonuclease Ⅰ excises the nucleotides of the daughter strand back to the site of the mismatch; 5: The daughter strand is resynthesized and the error is corrected.

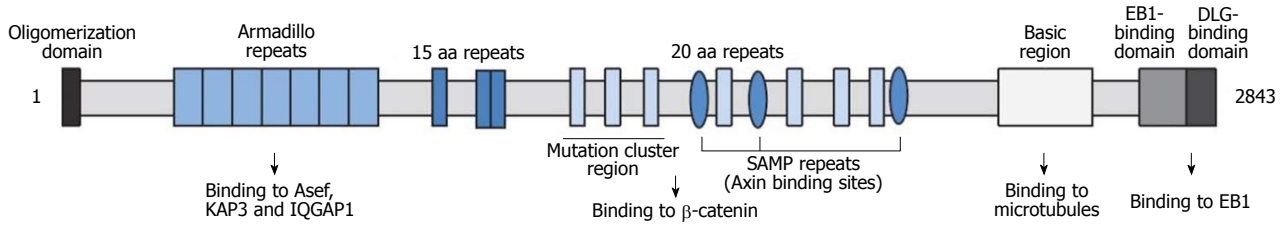

Figure 11 Major structure and functions of APC[35]. It is made up of oligomerization domain, armadillo repeats domain, 15-amino acid or 20-amino acid repeats domain, SAMP repeats domain, mutation cluster region, basic region, EB1-binding domain and DLG-binding domain.

domain conformational change and affect the hydrolysis of ATP or ADP to ATP conversion process. This change could disorder the open and close conformational transition of the MutSα complex and eventually lead to the loss of MMR expression (Figure 10).

The *APC* gene is located on the autosome 5q21. Its main functions include inhibiting cell proliferation, promoting differentiation, promoting apoptosis, participating in cell migration, cell adhesion and chromosome separation. It is generally believed that the mutation of *APC* gene is an important initiator for the occurrence and development of colorectal cancer[30,31]. Mutated *APC* can also be seen in some brain and breast cancers[32,33]. The molecular weight of APC protein is about 312 kDa, and its N-terminal domains mainly include: (1) Oligomerization domain; (2) armadillo repeats domain; (3) 15-amino acid or 20-amino acid repeats domain; (4) SAMP repeats domain and Mutation cluster region (MCR); (5) Basic region for binding microtubule or f-actin, including the 2219-2580th amino acids; (6) EB1-binding domain; (7) DLG-binding domain[34,35] (Figure 11). APC protein can participate in the composition of a complex promoting the destruction of β-catenin (a transcriptional permissive factor), and then negatively

regulate the classical Wnt signal pathway. Its C-terminal region, especially the 2200-2400th amino-acid, is rich in bases that could boost the binding process of microtubules[36-38]. APC protein of cell periphery plays an important role in binding microtubule network, establishing cell polarity and inhibiting invasion according to many studies[39,40]. A research based on a zebrafish model suggests that cell differentiation may be related to the Warburg effect mediated by *APC*[41]. In addition, the differentiation mechanism of human intestinal epithelial cells may be similar to that of zebrafish[42]. In this case, the mutation (c.6662T>C) causes 2221st methionine of the APC protein to become threonine, which may result in the protein conformation change, and affect cell differentiation, adhesion and migration.

In summary, we detected *MSH6* and *APC* gene mutations in a case of PJS by high-throughput sequencing technology, but failed to detect mutations in *LKB1/STK11* gene, and the mutation of *APC* gene (c.6662T>C: p.Met2221Thr) had never been reported previously. This suggests that mutations of *MSH6* and *APC* genes may affect the occurrence and development of PJS by affecting the MMR pathway, cell differentiation, adhesion and migration. Our research may be helpful in expanding the mutation spectrum of PJS and revealing its genetic heterogeneity.

ARTICLE HIGHLIGHTS

Case characteristics

A 32-year-old male patient with Peutz-Jeghers syndrome (PJS).

Clinical diagnosis

Peutz-Jeghers syndrome.

Endoscopic diagnosis

Multiple long pedicle, subpedicle or sessile polyps sized 0.5-1.2 cm appeared in the colorectum, especially the descending colon, sigmoid colon and rectum.

Imaging diagnosis

Many polyps of different sizes were distributed in the descending duodenum and the 3rd to 6th segments of the small intestine, and the local intestinal canal was torsional.

Treatment

The patient underwent a combined laparoscopic and endoscopic surgery.

Pathological diagnosis

PJS hamartoma polyps without canceration.

High-throughput sequencing

Instead of *LKB1/STK11* gene, we detected missense mutations of *APC* gene and *MSH6* gene.

Experiences and lessons

This research may be helpful in expanding the mutation spectrum of PJS and revealing its genetic heterogeneity.

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