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EDITORIAL OFFICE
Jin-Lei Wang, Director
Xiu-Xia Song, Vice Director
World Journal of Experimental Medicine
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381891
Fax: +86-10-85381893
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
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Baishideng Publishing Group Inc
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Telephone: +1-925-223-8242
Fax: +1-925-223-8243
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EDITORIAL

Impact and risk factors of post-stroke bone fracture

Kang Huo, Syed I Hashim, Kimberley LY Yong, Hua Su, Qiu-Min Qu

Kang Huo, Syed I Hashim, Kimberley LY Yong, Qiu-Min Qu, Department of Neurology, the First Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China

Hua Su, Center for Cerebrovascular Research, Department of Anesthesia and Perioperative Care, University of California, San Francisco, CA 94117, United States

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Correspondence to: Qiu-Min Qu, MD, PhD, Department of Neurology, the First Affiliated Hospital of Xi'an Jiaotong University, Yanta West road No. 277, Xi'an 710061, Shaanxi Province, China. quqiumin@medmail.com.cn
Telephone: +86-29-85324083
Fax: +86-29-85324083

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Abstract

Bone fracture occurs in stroke patients at different times during the recovery phase, prolonging recovery time and increasing medical costs. In this review, we discuss the potential risk factors for post-stroke bone fracture and preventive methods. Most post-stroke bone fractures occur in the lower extremities, indicating fragile bones are a risk factor. Motor changes, including posture, mobility, and balance post-stroke contribute to bone loss and thus increase risk of bone fracture. Bone mineral density is a useful indicator for bone resorption, useful to identify patients at risk of post-stroke bone fracture. Calcium supplementation was previously regarded as a useful treatment during physical rehabilitation. However, recent data suggests calcium supplementation has a negative impact on atherosclerotic conditions. Vitamin D intake may prevent osteoporosis and fractures in patients with stroke. Although drugs such as teriparatide show some benefits in preventing osteoporosis, additional clinical trials are needed to determine the most effective conditions for post-stroke applications.

Key words: Bone fracture; Recovery; Bone mineral density; Stroke; Osteoporosis

Core tip: Post-stroke bone fracture negatively impacts stroke recovery, prolongs hospital stays, and increases economic cost. Stroke, osteoporosis and bone fracture share common risk factors. The main risk factors for post-stroke bone fracture include aging, osteoporosis, and loss of posture control. Bone mineral density measurement may identify patients who are at risk of post-stroke bone fracture. Drugs and supplements, such as vitamin D and teriparatide, can be tested in clinical settings for prevention of post-stroke bone fracture. Although bisphosphonate's incur side effects, they are considered first-line drugs to prevent post-stroke bone fracture.

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INCIDENCE OF POST-STROKE BONE FRACTURE

Aging, smoking, hypertension and lack of physical activity contribute to osteoporosis bone fracture and stroke. Although strokes can occur at any age, they mostly occur in older age, which may due to reduction in bone density[1]. Only one fourth of strokes occur in people under the age of 65[2]. The prevalence of stroke more than doubles each decade after the age of 55.

Stroke and bone fracture share common risk factors. Individuals with a greater risk of stroke are also more prone to osteoporosis and fractures. Risk factors for post-stroke bone fracture include osteoporosis, aging, and loss of posture control. Osteoporosis is the main risk factor of bone fracture, with increasing prevalence worldwide. In the United Kingdom, over half of women, and one-fifth of men over age 50, will sustain a fragility fracture[3].

The risk of bone fracture post stroke is underestimated because it is often considered secondary to falling, especially among elderly. Previously, 534 elderly persons were assessed over 2 mo, results revealed that stroke was influenced by physical factors including falls[4]. A fracture post-stroke puts the patient at a greater disadvantage during rehabilitation, and greatly affects the quality of life after recovery.

RISK FACTORS OF POST-STROKE BONE FRACTURE

Stroke patients are highly susceptible to bone fracture[5]. They have a 2 to 4-fold increased risk of hip fractures, which carries a high mortality rate in the elderly[6]. Changes in walking posture, skeletal weight load, and bone maintenance have been studied as risk factors for poor post-stroke bone health[7]. Other factors include low bone mineral density (BMD), loss of balance, inappropriate gait, and muscle deformation[8]. In order to predict the risk of falls and fractures in stroke patients, we must take into account not only structural changes, but also functional disability.

Post-stroke fractures are of particular concern given the impact on functional outcomes of stroke rehabilitation. Functional impairment of stroke is commonly measured using two scales, the barthel index (BI) and modified Rankin scale (mRs). Both assessment scales place stroke patients with differing functional capabilities into categories, describing a level of independence or need for medical assistance. The BI is a measure of functional independence on ten activities of daily living (ADL). The mRs measures global disability of a patient. Harrison *et al*[9] studied the effectiveness and drawbacks of each scale and drew the conclusion that while all scales had weaknesses, certain types of disability were reliably assessed by these scales.

The level of functional impairment may be useful in estimating risk of falls. In a study of 135 elderly stroke patients, Nyberg *et al*[10] developed a fall prediction index that classifies patients into low-, intermediate-, and high-risk groups. The index includes male sex, poor ADL performance, urinary incontinence, bilateral motor impairment, impaired postural stability, visuospatial hemi-neglect, and use of diuretics, anti-depressants or sedatives as factors in increased falling[10]. A comprehensive index for assessing the risk of falls is vital to reliably predict the possibility of post-stroke bone fracture.

In addition, weak bones are a risk factor for post-stroke bone fracture. Hospitalization of stroke patients suffering adverse changes in motor function and body composition leads to long-term increase in fracture risk[11]. Although BMD decrease is common in stroke patients > 65 years, studies have demonstrated that BMD loss in paretic limbs are greater than their non-paretic counterparts, such as the arms. Interestingly, vitamin D deficiency causing osteopenia seems to be a possible mechanism[12]. Bone loss progresses rapidly in the acute stages of stroke[13], with the most dramatic changes seen in the first few months[14]. By measuring the magnitude of changes to volumetric BMD after stroke in a prospective study, Borschmann *et al*[15] found a 7% difference in total volumetric BMD between paretic and non-paretic distal tibiae 6 mo post-stroke, with lower density on the paretic side. Another study showed that while immobility does increase bone loss, stroke patients who maintained their ability to walk had a 3% decrease in BMD. This suggests that stroke itself is a risk factor for bone loss and fragile bones. Therefore, more studies are needed to better understand the influence and underlying mechanisms of stroke on BMD loss, as well as to develop preventive methods.

Biochemical markers related to bone turnover can be used to evaluate bone health and risk of bone fracture. Haddaway *et al*[16] showed that stroke patients older than 60 years displayed much earlier bone resorption (a process of bone breakdown) when compared with normal individuals, supporting the idea that bone remodeling is correlated with post-stroke bone fragility. Bone turnover in elderly women was inversely correlated with bone density at the hip on both hemiplegic and normal sides, which may adversely influence bone fracture. Serum biomarkers, such as osteocalcin (OC), bone alkaline phosphatase combined with BMD analysis better predict than physical exam alone. Early evaluation of bone turnover could help identify bone loss in stroke patients[17].

Motor changes, including posture, mobility and balance are also factors associated with bone loss. Chang *et al*[18] compared the femoral neck bone loss in hemi-

paretic patients with high or low weight-bearing on the paretic lower limb. The study showed that patients with lower standing weight placed on their paretic limb (< 50%) had faster bone loss and BMD reduction during the follow-up period (≥ 6 mo) compared with those who bore more weight (> 50%). The proportion of weight bore by the paretic leg was related to the BMD, indicating that reduced weight bearing is a predictor of bone loss in stroke patients[18]. Although results from a study by Melton *et al*[19] suggested that skeletal weight load is not an independent factor of bone loss, the effect of reduced skeletal weight load may be more pronounced in paresis. Immobility is also associated with increased bone loss in stroke patients, with wheelchair-bound patients showing a 4-fold loss in BMD (13% to 3%) compared with ambulating patients[20]. Together, these factors amplify the fragility of bones in patients undergoing rehabilitation, leading to osteoporosis[21].

Of course, some stroke patients are at a higher risk of having bone fractures than others. For example, a study by Ashburn *et al*[22] revealed that patients who were physically unstable (experienced near-falls), with upper-limb functional impairment, were at higher risk of falls. This suggests that proper arm function can prevent falls, as patients are able to support themselves using their arms. Interestingly, stroke survivors without functional impairment had significantly higher risk of fragility fractures in legs compared with arms. However, stroke survivors with functional impairment had no prevalence of fracture on any specific site of their body[23].

Prevention of fractures is of utmost concern, as post-fracture care can be long-term and strenuous, especially for stroke patients. Physical rehabilitation is essential for maximum motor recovery. Post-stroke rehabilitation includes re-learning of basic movement skills and other common activities. To perform daily activities, patients must recover balance, muscle strength, and movement. Early and consistent physical rehabilitation is key in achieving the most favorable results[24]. Having a fracture post or pre-stroke can put the patient at a greater disadvantage in their rehabilitation, and negatively affects their quality of life[25]. Bone fractures on the paretic side can be especially detrimental, as damage to the structure of the paretic limb complicates motor recovery.

There are many factors affecting the outcome of rehabilitation, including, but not limited to, the type of stroke lesion, age of the patient, previous lifestyle, rehabilitation, exercise and mobility[26]. Recent systematic reviews and meta-analyses show that in rat models, exercise was associated with decreased lesion volume and improved functional outcomes[27,28]. Studies also showed that early mobilization had a strong positive correlation with favorable outcomes in patients[29-31]. Despite the acceptance among studies on the positive effect of early mobilization, analyses of randomized-controlled trials comparing first-day and second-day mobilization on functional outcomes (good functional outcomes were mRs scores ≤ 2) showed little difference between the groups[32]. Future trials should define proper mobilization times at different functional impairment levels.

Patients with conditions that affect bone loss pre-stroke suffer more complications post-stroke. The effect of stroke on BMD has been independently observed in patients with pre-stroke osteoporosis[33]. Older women often experience bone loss after menopause, which is further exacerbated by stroke. In men, subjects with pre-stroke osteoporosis (due to aging or otherwise) suffer similar consequences of stroke, but given that most clinical trials have focused on menopausal women, male subjects are often neglected in studying osteoporosis.

The independent interaction of stroke on bone density may be difficult to observe due to the presence of multiple factors. Therefore, the multi-factorial character of post-stroke bone fracture needs to be studied deeper to determine possible interventions.

It is also interesting to note that there is a two-way interaction between stroke and bone health. Stroke leads to low BMD, and low BMD increases the risk of stroke[34,35]. A prospective investigation conducted between 1997 and 2000 evaluated the relationship between BMD and incidence of stroke in 14290 participants. The study showed that the risk of stroke increased in low BMD individuals. The findings indicated that BMD predicted the risk of stroke, especially in women[36]. It has also been shown that vertebral fracture occurs more often in first time-stroke patients that have low pre-stroke BMD. Therefore, low pre-stroke BMD poses a greater risk of fracture, resulting in additional functional loss[37]. Hypertension is also a major risk factor for stroke. Elderly persons with shorter stature and lower BMD have been associated with increased arterial stiffness and hypertension. Therefore, this population could be at higher risk compared with the general population for post-stroke bone fracture[38].

Low estrogen level is correlated with reduced BMD. At menopause, decreased estrogen leads to a rapid loss of BMD and increased incidence of bone fracture in women. Estrogen plays a key role in regulating bone mass and strength by controlling the activity of bone-forming osteoblasts and bone-resorbing osteoclasts[39]. Therefore, post-menopausal women are more likely to develop osteoporosis, and thus have an increased risk of bone fractures. According to statistical data obtained from the International Osteoporosis Foundation, osteoporosis causes more than 8.9 million fractures worldwide annually, resulting in an osteoporotic fracture every three seconds[40].

Using anti-depressants on stroke patients may increase the possibility of post-stroke bone fracture. Stroke patients commonly suffer from secondary disorders, such as depression, that may affect bone health. Anti-depressants, in particular selective serotonin reuptake inhibitors (SSRIs), have been correlated with an increased risk for osteoporosis[41]. The association of fracture incidents and

antidepressants was studied using data from the Canadian Multicenter Osteoporosis Study in a prospective randomly selected population-based community cohort[42]. Among 6645 subjects, 192 (2.9%) were using SSRIs and/or serotonin and noradrenaline reuptake inhibitors (SNRIs) at baseline. During the 10-year study period, SSRI/SNRI use was associated with increased risk of fragility fracture (HE = 1.88; 95%CI: 1.48-2.39). Therefore, more attention and care should be given when prescribing antidepressant drugs to patients.

Although current evidence on the impact of stroke on bone health seems contradictory, what is clear is that risk of bone fracture and stroke are strongly correlated. However, whether stroke is an independent factor for BMD loss, due to motor function changes, is unknown. To prevent bone fracture, methods and treatments to reduce bone loss in stroke patients should be researched. A clear time frame for physical therapy, especially early mobilization, is important in long-term recovery. Patient fall prediction indexes can be developed further, and rehabilitative measures would function better when tailored to different risk grades. Ultimately, while evidence strongly supports the effectiveness of general rehabilitation, a deeper understanding of treatment response should be prioritized.

ASSESSMENT OF POST-STROKE BONE FRACTURE

In this section, we discuss treatment options available today as well as assessments to minimize the risk of bone fractures and to optimize post-stroke recovery. Given that osteoporosis is a predictor for stroke and bone fractures, the majority of the following discussion is focused on management of this condition.

Calcium is a major component of bone, supplementation of calcium is regarded as a possible solution for preventing post-stroke bone fracture. However, there is conflicting data on the effectiveness of calcium supplementation. Some evidence shows calcium intake has adverse cardiovascular effects, raising widespread concern. However, a recent study showed dairy products do not increase the risk of cardiovascular disease, particularly low fat versions. Therefore, dairy products could be a good resource for calcium intake[43].

Recent studies have shown that increasing the intake of calcium does not reduce osteoporotic fracture rates[44]. A prospective longitudinal cohort study revealed that gradual increases in dietary calcium intake above the first quintile in female population were not associated with further reductions to the risk of fracture or osteoporosis[45]. If these findings are true, then taking calcium will not prevent osteoporosis. Instead, it may increase the risk of developing cardiovascular problems long term.

Alternatively, other studies have shown that calcium supplementation does not increase carotid artery intimal medial thickness or carotid atherosclerosis[46]. Thus, the impact of calcium intake on osteoporosis is not clear. Until more confirmatory data are available, physicians should not stop prescribing calcium supplements to their patients[47]. The appropriate intake amount varies, ranging 800-1500 mg/d[48]. Some sources recommend calcium supplementation not exceed 1200-1500 mg/d. A study by Khan *et al*[49] found that to decrease fracture, non-fatal CVD, stroke, and all-cause mortality risks in both men and women, a specific dose of 1348 mg/d is optimal.

Given no clear consensus on proper calcium dosage, early screening and active management of osteoporosis at the acute stages of stroke are critical[37]. One of the best predictive factors for osteoporosis is BMD, and it should be measured on all stroke patients. Dual-energy X-ray absorptiometry (DXA) is a primary tool that accurately evaluates BMD. According to the United States Preventive Services Task Force, women over the age of 65 should get a DXA scan, men are recommended to get the scan at age 70[50]. At risk individuals should also get the scan performed. Risk is determined using the World Health Organization' s Fracture Risk Assessment Tool, a diagnostic that evaluates the 10-year probability of bone fracture risk. Clinical risk factors assessed include prior fragility fracture, parental history of hip fracture, current tobacco smoking, long-term use of glucocorticoids, rheumatoid arthritis, other causes of secondary osteoporosis, and daily alcohol consumption[51].

There are 2 DXA scan methods, a central DXA that scans the hip and lower spine, and a peripheral DXA that most often scans the heel. A study showed the heel BMD can serve as a surrogate for hip BMD[52]. BMD measurements, used concomitantly with the Tinetti Test (TT), or Performance Oriented Mobility Assessment, provide a useful indication for those needing early prophylaxis against bone loss. The TT is a solid predictor to assess balancing ability of post-stroke patients. It was created to screen for balance and mobility skills (gait) in older adults, and determines the likelihood of falls. This is especially important in hemiplegic stroke patients. Using a 3-point scale, a score less than 19 indicates a high risk for falls, and scores between 19 and 24 indicate a moderate fall risk[53]. However, it is difficult to assess with specificity on a 3-point scale[54]. This test is not useful on post-stroke patients that are bed-ridden.

A more reliable assessment is the Timed "Up and Go Test" (TUG). It is the shortest, simplest clinical balance test. A stop-watch timer is more objective compared with a rating scale used in the TT[55]. In a cross-sectional study performed by Ng and Hui-Chan, the TUG test showed excellent reliability (ICC > 0.95). Subjects with chronic stroke were found to have more significant spastic and weaker plantar flexors, slower walking speeds, and poorer walking endurance when compared to healthy elderly subjects (all $P < 0.003$)[56].

With the balancing ability assessed by either the TT or TUG, the severity of disuse on the hemiplegic side of

post-stroke patients can be determined. This disuse of the hemiplegic side in stroke patients results in bone-mass reduction, especially in the presence of vitamin D deficiency[57]. By comparing BMD, serum concentrations of intact parathyroid hormone (PTH), OC, tartrate-resistant acid phosphatase, 25-hydroxyvitamin D (25-OHD), and calcium levels between healthy and post-stroke patients, BMD values were lower on the hemiplegic side compared with the non-hemiplegic side. Vitamin D deficiency and compensatory secondary hyperparathyroidism stimulated skeletal turnover is an important cause of osteopenia in the hemiplegic limbs of stroke patients. This suggests that administration of vitamin D supplements is beneficial in stroke patients to reduce fracture risk.

In addition to increasing risk of bone fractures, vitamin D deficiency in post-stroke patients may also cause a variety of issues in non-stroke patients. Vitamin D deficiency impairs gastrointestinal absorption of calcium and bone mineralization, muscle strength, and is also associated with muscle mass loss, which contributes to an increased risk of fall[58]. Furthermore, vitamin D deficiency is a risk factor for strokes due to the associations of low 25-OHD levels, particularly in the presence of arterial hypertension[59]. Vitamin D also exhibits neuroprotective, as well as neuromuscular and osteo-protective effects, which may reduce cognitive and functional impairments in stroke patients. However, current evidence is too scarce to draw any conclusions. Further evaluations are needed to confirm the effect of vitamin D treatment in reducing stroke associated mortality and morbidity.

While vitamin D supplementation is important in patients who have osteoporosis, there is conflicting evidence showing that vitamin D does not prevent osteoporosis in healthy individuals. In a meta-analysis from 2014, the effects of vitamin D supplements on BMD were found to have no significance[60]. However, some studies showed that vitamin D with co-administration of calcium significantly improved BMD and thus, reduced the risk of falling in elderly patients[61].

Another strong indicator for osteoporosis is serum OC, which is associated with bone turnover. By examining serum total osteocalcin (TOC), carboxylated osteocalcin (COC), and their ratio (COC/TOC), data show serum COC concentrations, especially COC/TOC, predicted the occurrence of fractures in the elderly[62]. Thus, low COC/TOC is directly correlated with risk of fracture. Bone turnover can be further evaluated using serum urinary cross-linked N-telopeptide of type Ⅰ collagen (NTX), a reliable bone resorption marker in patients with metabolic bone disease. According to a study performed by Maeno *et al*[62], patients in the highest quartile of serum NTX concentrations exhibited rapid bone loss rates, with a sensitivity and specificity for detecting the rate of 48% and 83%, respectively.

Therefore, assessment instruments, such as the TT, TUG test, DXA scan, serum NTX, and COC/TOC levels, as well as supplements such as calcium and vitamin D, are important in identifying and preventing osteoporosis and fractures in patients with stroke. If preventive measures against osteoporosis are not well managed, or if a person already has osteoporosis, the treatment goals should be to minimize further bone loss and prevent osteoporotic fractures.

PREVENTION AND ADDITIONAL MANAGEMENT

Osteoporosis is a devastating disorder that impairs bone strength, causing an increased risk of fracture. It is usually diagnosed by assessing BMD *via* the DXA scan, and is defined by the World Health Organization criteria as a BMD T-score of 2.5 standard deviations or more below the average of a young, healthy person[63].

Most studies show that mitigation of osteoporosis prevents post-stroke bone fracture. Hemiplegic patients and severe stroke patients will experience bone remodeling. A study by Ramnemark *et al*[64] in 1999, with 24 extensive paresis stroke patients, found that during the first year after severe stroke, patients developed pronounced hemi-osteoporosis. This was not associated with general changes in lean or fat mass. The development of hemi-osteoporosis was independent of weight changes after stroke[65].

Currently, bisphosphonates are used to treat osteoporosis because these drugs slow bone breakdown due to their strong affinity to skeleton, low toxicity to other tissues and organs in the body, and ease of frequency of administration[66]. Alendronate, risedronate, ibandronate, and zoledronate were found to reduce fracture risk by 50% within the first year of therapy in both men and women[67]. However, some studies suggest that bisphosphonates may increase the risk of esophageal cancer and cardiovascular diseases[68], osteonecrosis of the jaw, and subtrochanteric fractures[69,70]. These side effects seem likely to hinder the use and benefits of bisphosphonates. Thus, bisphosphonates use should be determined on a case-to-case basis.

Recent studies show that teriparatide, a synthetic peptide that mimics the action of PTH, rebuilds bone and reverses osteoporosis. A large, randomized, placebo-controlled clinical trial found that postmenopausal women with severe osteoporosis had reduced spinal and non-vertebral fractures by more than 50% when using teriparatide[71]. However, this trial was stopped after controversy that teriparatide caused osteosarcoma, a type of bone cancer, when injected into laboratory rats[72]. Retrospective studies have found no significant association between osteosarcoma and teriparatide, and only 2 cases of osteosarcoma in PTH-treated patients have been reported. The prevalence of osteosarcoma in rats is likely due to the ever-growing epiphysis in rats. Thus, teriparatide is contraindicated in children and young adults whose epiphysis has not closed. Adults that are pregnant, have risk of renal failure, or have a history of radiation therapy should also refrain from using teriparatide. Currently, teriparatide should be limited to 2 years, and is not encouraged to prevent osteoporosis,

to treat mild osteoporosis, or by people who can take other osteoporosis treatment[73]. The post-treatment effect of bone loss is prevented by adding an anti-resorptive drug after stopping teriparatide. Based on the researches we already have, benefits of bisphosphonates surpass disadvantages for not using them. Regardless of bisphosphonate's side effects, it is still a first-line drug choice to prevent post-stroke bone fracture. However, large scale randomized double blind clinical trials still should be conducted in the further to explore a safe preventive method for reducing bone fracture on post-stroke patients.

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FRONTIER

Inflammatory diseases modelling in zebrafish

Camila Idelí Morales Fénero, Alicia Angelina Colombo Flores, Niels Olsen Saraiva Câmara

Camila Idelí Morales Fénero, Niels Olsen Saraiva Câmara, Laboratory of Transplantation Immunobiology, Department of Immunology, Institute of Biomedical Sciences IV, University of São Paulo, Cidade Universitária, São Paulo 05508-900, Brazil

Alicia Angelina Colombo Flores, Laboratory of Neuronal Circuit and Craniofacial Development, Anatomy and Developmental Biology Program, ICBM, University of Chile, Santiago 8380453, Chile

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Correspondence to: Niels Olsen Saraiva Câmara, MD, PhD, Titular Professor, Laboratory of Transplantation Immunobiology, Department of Immunology, Institute of Biomedical Sciences IV, University of São Paulo, Cidade Universitária, São Paulo 05508-900, Brazil. niels@icb.usp.br
Telephone: +55-11-30917388
Fax: +55-11-30917224

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Abstract

The ingest of diets with high content of fats and carbohydrates, low or no physical exercise and a stressful routine are part of the everyday lifestyle of most people in the western world. These conditions are triggers for different diseases with complex interactions between the host genetics, the metabolism, the immune system and the microbiota, including inflammatory bowel diseases (IBD), obesity and diabetes. The incidence of these disorders is growing worldwide; therefore, new strategies for its study are needed. Nowadays, the majority of researches are in use of murine models for understand the genetics, physiopathology and interaction between cells and signaling pathways to find therapeutic solutions to these diseases. The zebrafish, a little tropical water fish, shares 70% of our genes and conserves anatomic and physiological characteristics, as well as metabolical pathways, with mammals, and is rising as a new complementary model for the study of metabolic and inflammatory diseases. Its high fecundity, fast development, transparency, versatility and low cost of maintenance makes the zebrafish an interesting option for new researches. In this review, we offer a discussion of the existing genetic and induced zebrafish models of two important Western diseases that have a strong inflammatory component, the IBD and the obesity.

Key words: Zebrafish; Western diseases; Inflammatory disorders; Obesity; Inflammatory bowel diseases

Core tip: The western lifestyle with a high fat and carbohydrates diet, lack of physical activity and stress, is a trigger for different diseases with complex interactions between the host genetics, the metabolism, the immune system and the microbiota, as the inflammatory bowel

disease (IBD), obesity and diabetes. The zebrafish has 70% homology with our genes, shares anatomic and physiological characteristics with mammals, and emerges as a new model for the study of metabolic and inflammatory diseases. In this review, we examine the existing genetic and induced zebrafish models of two important Western diseases with strong inflammatory component, IBD and obesity.

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INTRODUCTION

In the last decades, the standard of living of the western world has had consequences that affect human health. Factors such as a diet that is high in carbohydrates and fats, a sedentary lifestyle, and stress trigger a state of chronic systemic low-grade inflammation, insulin resistance, and changes in the microbiota[1-3], which lead to the so-called Western diseases. Some of these diseases include inflammatory bowel disease (IBD), obesity, type 2 diabetes and heart disease, among others, and they are an issue of global significance, because of the high incidence of such disorders in western society. It is estimated that IBD affects approximately 1-1.3 million people in the United States[4] and in the same country 9.3% of the population has diabetes[5]. Additionally, more than two-thirds (68.8%) of United States adults are considered as overweight or obese[6].

Given the worldwide importance of these diseases, much research is currently in progress seeking answers to unresolved questions about their physiopathology, the pathways involved and new therapies to treat these conditions. Although mainly mammalian models, such as rabbits, rats and mice, are used for these studies[7-10], other models that have been gaining ground in the field of inflammatory diseases do exist[11-13]. The zebrafish is a small tropical freshwater fish primarily used as a vertebrate model in developmental biology because of its characteristic high fecundity, short *ex vivo* development time, ease of observation in the embryonic and larval states, relative ease of genetic manipulation, and low cost of production[14]. Additionally, the zebrafish genome is fully mapped (http://www.sanger.ac.uk/resources/zebrafish/), having approximately 70% of orthologs with the human genome[15]. Zebrafish have specific technical advantages as model for the *in vivo* analysis and knock-down technology[12,16]. They have anatomical features commonly found in mammals, including a central, autonomic and enteric nervous system, a multi-chambered heart, a liver, an intestinal system, a pancreas, and a kidney responsible for the production of hematopoietic cells, as well as immunological maturation sites such as the thymus and the spleen[13]. Zebrafish have a functional innate immune system at 48 h post-fertilization (hpf) and a mature adaptive system approximately 4-6 wk post-fertilization (wpf)[17], with many of the same immune cells, cytokines and chemokines known in humans[18]. Furthermore, in the last decade, zebrafish have become a model for different human diseases and a tool for drug screening[11,13,19]. All of these characteristics make the zebrafish an excellent model for the study of inflammatory pathologies.

In this review, we discuss and summarize the current larval and adult zebrafish models of Western diseases with an inflammatory component, including IBD and obesity.

IBD MODELS

In humans, the IBD are a group of chronic inflammatory conditions of the small intestine and colon, appearing as a result of deregulated interactions between the immune system and the commensal microbiota, triggered by a genetic predisposition of the affected individual and external factors[20-22]. In mice, there is a wide range of genetic, spontaneous and chemical models[23-25] that have been used in an attempt to find answers to different about IBD issues. Beginning some years ago, an increasing variety of zebrafish IBD chemical models have arisen, which are based on models previously tested in mice.

The zebrafish intestine has been described by many authors[26-28] as very similar in anatomy and architecture to the mammalian small intestine. It is a compartmentalized tubular structure with three intestinal segments defined by histological morphology of the epithelial folds and the distribution of different cell types. It has a mucosal layer of simple folded epithelium formed by columnar absorptive enterocytes, goblet cells and enteroendocrine cells; it lacks Paneth cells and a lamina propria beneath the epithelium. The mucosa is directly surrounded by circular and longitudinal smooth muscle layers, and small groups of enteric nerves can be observed between the two muscular layers with the nerve fibers innervating the connective tissue beneath the epithelium. Because of these simple anatomical characteristics, the zebrafish has proven to be an excellent model to study intestinal inflammation.

Genetic susceptibility

Nowadays, there's little evidence of genetic spontaneous colitis models in zebrafish as exist in mice [*e.g.,* NEMO KO, STAT3 KO in myeloid cells, interleukin (IL)-10 KO][29-31], however, there have been discovered many zebrafish genes related to genetic susceptibility in human IBD. The loss of *myd88*[32,33], an adaptor molecule, central in innate immune signaling[34], induce predisposition to bacterial infections and compromised expression of immune transcription factors (*nfκb, ap-1*) and molecules (*il-1β, mmp9*), proving to be an important molecule in the development of the inflammatory process in zebrafish. NOD proteins are intracellular pattern recognition receptors involved in innate immune response and have been associated with genetic vulnerability to IBD[35].

In zebrafish, *nod1* and *nod2* genes are expressed in intestinal epithelial cells (IECs) and neutrophils. In a model of infection with *Salmonella enterica,* morpholino (MO) knock-down (KD) of *nod1* and *nod2* have decreased survival after infection, and *nod1* KD also had a decreased expression of dual oxidase (*duox*), which is responsible for the synthesis of reactive oxygen species and has role in neutrophils migration, since its depletion slows down the repopulation of the caudal hematopoietic tissue[36]. In mammals, the cytokine IL-22, which is produced by T-helper cells and innate lymphocytes, has important functions in host defense at mucosal surfaces and in tissue repair[37]. In zebrafish, IL-22 expression was detected predominantly in the myeloid innate linage during early developmental stages, and proved to have participation in host-microbe interaction since its knock-down present high susceptibility to bacterial infections and an increased pro-inflammatory cytokine expression. IL-22 is increased in patients with Crohn's disease (CD) but is decreased in ulcerative colitis (UC) patients[38,39], thus would be interesting investigate the role of this cytokine during zebrafish intestinal inflammation. Furthermore, IL-10 and IL-23 expression have been related to an upregulated response to LPS and bacterial infection in zebrafish[40,41], and both have proven roles in IBD, since the *il10*$^{-/-}$ mice develops a spontaneous colitis[31] and IL-23 is essential for T cell–mediated colitis[42].

Endoplasmic reticulum (ER) stress is a defense mechanism triggered by a variety of conditions that disturb folding of proteins in the ER. To alleviate this stress the unfolded protein response (UPR) is activated, restoring ER homeostasis, promoting cell survival and adaptation. Modifications in genes that are centrally involved in the UPR appears as a risk factor for both forms of IBD, UC and CD[43]. In zebrafish, two mutants present ER stress in IECs, the *sec13*^{sq198} and *cdipt*^{hi559}, with defects in intestinal development in the first one and alteration of the villi, disorganization in the proliferation of IECs, apoptosis of goblet cells, abnormal mucosecretion, bacterial overgrowth and leucocyte infiltration, in the second one, characteristics resembling the IBDs[44,45]. All these examples, demonstrates conserved genes and pathway in zebrafish, that makes it an interesting model for the research of new IBD related genes.

Chemically induced adult models

Disruption of the intestinal epithelial barrier and exposition to luminal bacterial antigens into the mucosa is one of the key characteristic of mammalian IBD[21], and is the main accomplishment of most of the chemicals used to induce colitis, closely resembling morphological, histopathological and symptomatical features of human IBD. The first chemical model of intestinal inflammation studied in adult zebrafish[46] was based on the mouse oxazolone model of UC[47]. A concentration of 0.2% Oxazolone/50% ethanol was intrarectally injected inducing an inflammation characterized by the intestinal infiltration of granulocytes, eosinophils, macrophages and lymphocytes, as well as changes in the intestinal architecture such as bowel-wall thickening, loss of intestinal folds, and depletion of goblet cells. An increase of *tnfα*, *il1β*, and *il10* transcripts was also observed and was reversed by the use of antibiotics prior the induction of the colitis. A marked influence of the microbiota was evidenced by an enhanced susceptibility to inflammation due to an increase in the bacterial load when fishes where kept in stand-alone/static tanks than in continuous flow-tanks. Treatment with vancomycin, an antibiotic active against gram-positive bacteria, resulted in the reduction of the enterocolitis score and the infiltration of neutrophils, as well as an outgrowth of the *Fusobacteria* phylum; while treatment with colistin, targeting gram-negative bacteria, did not affect the total enterocolitis score but reduced eosinophilic and lymphocytic infiltration and increase in Proteobacteria group. This indicates that the oxazolone model in zebrafish can be used as a complementary model for the study of experimental UC.

A second model of intestinal inflammation in adult zebrafish used 2,4,6-trinitrobenzenesulfonic acid (TNBS) in a 30% ethanol solution injected intrarectally[48]. In mice, this model is widely used to study IBD because of clinical and histopathological findings resembling those seen in CD[49,50]. Using a wide range of TNBS concentrations, the authors observed a reduction of fish survival in a dose-dependent way with a recovery in survival rate when fishes were treated with vancomycin. A histological analysis showed disruption of the epithelial integrity with presence of ulcerations, swelling, thickening and the detachment of villi. No changes in goblet cells were observed in TNBS treated fishes. Inflammatory events peaked at 6 h post-induction (hpi), with an increase in infiltrated neutrophils in Tg(*mpx:eGFP)* animals and a significant increase in mRNA expression of *il1β, il8* and *il10,* in TNBS-exposed fishes compared with controls. These results are similar to a murine TNBS model in which an increase in neutrophilic infiltration in damaged tissue associated with a high myeloperoxidase activity[51] and an increase in TNFα levels[10] can be observed. Melanine-Concentrating Hormone (MCH) is a conserved neuropeptide involved in appetite regulation that recently has been related with intestinal inflammation[52]. The *mch2* gene isoform, equivalent to mammalian *mch*[53], was upregulated in the intestine in TNBS-exposed zebrafish; however the *mch1* isoform did not show any change. The MCH receptor MCHR1b was also upregulated, while MCHR2 was downregulated after TNBS treatment. This downregulation of MCHR2 is different than the expression of MCHR2 in humans, which was shown to increase during intestinal inflammation. Further studies are necessary to discover the contribution of MCH to intestinal inflammation.

The oxazolone and TNBS adult zebrafish enterocolitis models are comparable with the respective murine models in some aspects and provide a complementary tool for the study of IBD. However, because these models have been recently developed, studies that delineate all their inflammatory and pathologic features in order to

enhance their use are scarce in the literature, and more studies are necessary to accomplish this goal.

Chemically induced larval models

Contrary to adult models, the larval model lacks a functional adaptive immune system[17,54]; therefore it allows the observation of the isolated participation of innate immunity in inflammatory intestinal conditions. Additionally, the use of a larval model permits exploitation of transgenic lines to visualize *in vivo* changes in digestive organs and immune cells, such as gutGFP[55], Tg(*mpx:EGFP*)[114], Tg(*ptprc:DsRed*)[sd3][56], MO microinjections[57] and the CRISPR/Cas9 system[58], along with mutagenesis screening to discover novel candidate genes involved in diseases, among other applications.

The most used colitis model in zebrafish larva was developed by two different research groups[59,60] using TNBS in concentrations between 50-75 μg/mL diluted directly in the swimming water of embryos at 3 dpf to 6-8 dpf, higher concentrations resulted in less than 50% survival 3 d post-exposure[60]. Different histological characteristics were observed depending on the time and concentration of the TNBS exposition. Several changes were shown for a treatment of 5 d and 75 μg/mL TNBS including an expansion of the intestinal lumen, a smoothing of the epithelial line, a loss of villi and epithelial clefts, and an increase in the number of goblet cell throughout the mid and posterior intestine[59], with first changes appearing at 6 dpf[61]. Whereas, a 3 d exposition of 50 μg/mL TNBS does not induce any change in intestinal cell morphology or increase in goblet cell number[60]. We think that discordance on these characteristics in different research groups could also be influenced by the variability of the microbiota in the different facilities[62], nevertheless, more studies need to be conducted to test this hypothesis. Subcellular changes in TNBS-exposed larvae included the accumulation of lysosomes in the apical region of the epithelial cells and the loss of tight and gap junctions between IECs[59], as observed in human IBD[63,64]. A well preserved microvilli were present in both TNBS and controls larvae, suggesting a direct action of the TNBS on the physiology of the IECs and not an erosive action. TNBS-exposed fishes showed increases expression of *il1β*, *tnfα*, *il8*, *il12a*, *infg1-2*, *il10* at 6 dpf in the intestinal tissue[60] (Morales Fénero CI *et al*, unpublished data). Interestingly, some of these molecules are Th1 type cytokines, and though the larvae lacks circulating lymphocytes, they are probably produced by epithelial and/or infiltrating myeloid cells[65,66], as observed in the intestine of Tg(*mpx:EGFP*; *ifabp:RFP*)[60] and Tg(*lys:DSRED*) TNBS-exposed larvae (Morales Fénero CI *et al*, unpublished data). Moreover, an increase in TNFα expression in the intestinal lumen was directly related to the TNBS dose and could be reverted with a prednisolone treatment[59,61]. Decrease in RNA expression of ileal fatty acid binding protein *(fabp6)*[67] and increased intestinal lipids accumulation visualized by Nile red (NR) lipophilic stain, reflect alterations in fatty acid metabolism in TNBS-treated fishes, as well as, loss in the endocytic function in the mid-intestinal region[68]. Furthermore, a slight disruption of the intestinal vasculature was evidenced by a reduction of intestinal capillary branches and a decrease in the expression of vascular endothelial growth factor, in colitis induced larvae.

The microbiota is a source of pathogen-associated molecular patterns against which the immune cells can react and is a principal factor in IBD pathology[22]. Broad-spectrum antibiotics increased the low survival rate obtained with high concentrations of TNBS, and even recover the low survival rate of TNBS-exposed *myd88* morphants to control levels[60]. In addition, TNBS exposition in larva induced a lesser diversity of bacteria, with an increase in the phylum Proteobacteria (*Hydrocarboniphaga daqingensis*, *Limnobacter sp.*, *Citrobacter freundii*, *Comamonas sp.*, *Salmonella sp.)* and a decrease in the phylum Firmicutes (*Lactococcus plantarum* and *Streptococcus sp.*) compared with the control group[61]. The same bacteria phyla have been previously observed altered in human IBD[69,70]. Finally, treatment with 5-aminosalicylic acid co-administered with TNBS, prevented the disease alterations induced by the hapten, decreased the expression of *il1β, tnfα, ccl20* and *il8,* and inhibited the increase of myeloid cells in (*lys:EGFP)* transgenic larva, as well as reduced the recruitment of leucocytes to the intestine and skin in Tg(*mpx:EGFP*) larvae[59,60]. Similar results were obtained with prednisolone which was also effective against the generation of disease changes, including reduced expression of *il1β, tnfα* and *il8*[60], a decrease in TNFα and reduction of the number of goblet cells to normal levels[59]. Treatment with NOS inhibitors, rescued the *in vivo* and histological disease phenotype, while treatment with the immunomodulatory drugs thalidomide and parthenolide failed to rescue the changes induced by TNBS despite a decrease in the expression of TNFα in the intestinal tissue. These results indicate that the zebrafish can be an excellent pharmacological tool for drug screening in the search for new treatments for inflammatory diseases.

An additional murine version of UC model uses Dextran Sulfate Sodium (DSS) in the drinking water in an acute protocol of 5-7 d[9,71] or with the induction of chronic inflammation interspersed with periods of DSS-water and periods of recuperation with normal water[72,73]. In another attempt to use the zebrafish as a model for intestinal diseases, researchers exposed larvae at 3 dpf to 0.5% DSS for 3 d, generating a phenotype of marked mucus production with no changes observed in the number of goblet cells in the mid-intestine and the esophagus[74]. Nevertheless, exposure to DSS did not affect the expression levels of the *muc* gene, an ortholog of the human MUC5 gene family that is expressed in the esophagus. However, other "*muc* genes", such as *muc2.1,* which is highly expressed in the gut can also be analyzed for changes in this model[75]. An analysis of *Tg (kita:GAL4, UAS:EGFP)* larvae that labels fin fold mucus producing cells revealed a slight increase in the number of positive

cells when the larvae were treated with DSS, compared with controls[76]. Suggesting that intestinal goblet cells and not esophagus goblet cells, are altered and that they are responsible for the production of excess mucus. An augmented bacterial load and an increased number of intestine-infiltrating neutrophils was observed in *Tg(mpx: EGFP)^{i114}* DSS-exposed larvae compared with controls. This was reversed by antibiotic and dexamethasone treatment, however, depletion of the microbiota prevented the appearance of the DSS-induced mucosecretory phenotype while dexamethasone did not have the same effect. Exposition to DSS also increased the levels of *ccl20, il1β, il23, il8, mmp9* and *tnfα,* and decreased the proliferating cell nuclear antigen *(pcna)* gene. Curiously, using DSS at lower concentrations (0.25%) caused a loss of the inflammatory characteristics but a persistence of the mucosecretory phenotype, which was protective against TNBS induced colitis, and could be suppressed with a retinoic acid treatment, resulting in a worse survival rate and increased neutrophil infiltration.

Based on the premise that the use of non-steroidal anti-inflammatory drugs (NSAIDs), could lead to the impairment of mucosal barrier function[77,78], researchers used the NSAID glafenine as an inducer of intestinal inflammation[79]. An overnight treatment of 12 μmol/L glafenine in 5 dpf larvae produced an obstruction of cells and debris in the lumen of the mid-intestine and posterior intestine of the zebrafish, which consisted of dead IECs. Analysis of transversal sections showed intestinal shedding and an obstructed lumen with hypertrophic and hyperplasic IECs, with apical-pyknotic nuclei, sings of cellular damage and apoptosis, which were confirmed by an increase in activated caspase-3 positive cells. The visualization of glafenine-treated fish with transmission electron microscopy showed apoptotic IECs, cellular debris and microvesiculation of IECs, as well as, a pitted ER and organelles enveloped by membranes, which are signs of ER stress[80]. This characteristics were reverted by treatment with the μ-opioid receptor agonist (D-Arg2,Lys4) dermorphin-(1,4)-amide (DALDA), which decreased the formation of debris and apoptotic cell obstruction, improved the survival of glafenine-exposed larvae, decreased the expression of caspase-3, and increased the number of proliferative 5-ethynyl-2′-deoxyuridine (EdU) positive cells. Furthermore, DALDA-treated larvae showed decreased ER stress in IECs and a conserved epithelial architecture, as well as, upregulation of the UPR mediators spliced-xbp1 *(s-xbp1)* and activating transcription factor 6 (*atf6*), which were normal in glafenine-exposed animals. For other side, *atf6*-MO suppressed the rescue mediated by DALDA. This is anomalous with the function of the mammalian ATF6, which has apoptotic effects[81]. A lack of study on immune parameters such as innate cells infiltration or the quantification of cytokines and chemokines, leaves us with little knowledge about this model, which could be an excellent model to study of ER stress during intestinal inflammation. Modifications in genes that are centrally involved in the UPR appear to be a risk factor for both forms of IBD, UC and CD[43-45].

The aforementioned models of intestinal inflammation, summarized in Table 1, encompass the current options to analyze different aspects of IBD, including the possibility of study in conditions of isolated innate immune system. Other tools, as the generation of gnotobiotic zebrafish[82,83] make this good model system for the study of the microbiota that is central to the pathology of IBD.

OBESITY AND METABOLIC SYNDROME

Another western-lifestyle disease that has worldwide impact and involves chronic inflammation is obesity and the associated metabolic syndrome. Subjects related to lipid metabolism in zebrafish are relatively new but have begun to gain ground in the study of adipogenesis, metabolic alterations and obesity.

Zebrafish, as other teleosts, are poikilothermic animals and they only have white adipose tissue (WAT) and lack brown fat, which is more characteristic of homoeothermic organisms. The first visceral adipocytes appear to form in proximity to the pancreas after exogenous feeding is initiated, and they increase in number and distribution as the zebrafish grow, with the participation of the markers of adipocyte lineage peroxisome-proliferator activated receptor γ (*pparγ*) and fatty acid binding protein 11a (*fabp11a*)[84]. Lipid absorption in zebrafish is very similar to the process in mammals. Bile is synthesized in the liver, stored in the gall bladder and brought to the intestine through the bile duct, where it emulsifies lipids that are broken down by luminal lipases and absorbed like fatty acids and triacylglycerols. Lipids are transported in the plasma as unbound fatty acids or bound to carrier proteins, as triacylglycerols (TAG)-rich chylomicrons, and then they are delivered to the liver and stored in visceral, intramuscular and subcutaneous reservoirs, mainly as TAG[85-87]. Currently, a variety of techniques exists that allows visualization of the lipids in zebrafish without sacrificing the animal, including the fluorescent compounds NR and BODIPY® - conjugated lipids and Oil Red O, which is suitable for fixed fish and is an excellent tool for the study of lipid metabolism[86].

Genetic models of obesity

Energy homeostasis in the zebrafish is conserved and regulated by peripheral signals like PYY, GLP-1, ghrelin, adiponectin, leptin and insulin[88-93], originated in the gastrointestinal tract and adipose tissue, processed in the brain by the central melanocortin system (CMS), as in mammals[86,94,95]. The CMS circuits include the pro-opiomelacortin (*POMC*) gene, melanocortin peptides and its receptors (MC1R-MC5R), as well as the melanocortin antagonist agouti-related protein (AgRP)[96,97]. AgRP mRNA is upregulated by fasting in humans, mice and zebrafish[97-100], and its overexpression has been related to obesity in mice[101]. Song *et al*[102] created a transgenic animal that overexpressed the *AgRP* gene under the control of the *β-actin* promoter. AgRP transgenic animals were demonstrated to gain more weight, present

Table 1 Zebrafish models of inflammatory bowel diseases

Model	Age	Induction	Characteristics
Oxazolone	Adult	Intrarectal administration of 0.2% oxazolone in 50% ethanol. Stand-alone tanks	Epithelial damage; infiltration of neutrophils and eosinophils in intestine; depletion of goblet cells; upregulation of IL1β, TNFα and IL10[46]
TNBS	Adult	Intrarectal administration of TNBS (160 mmol/L) in 30% ethanol. Stand-alone tanks	Dose-dependent fish survival; disruption of the epithelial integrity; ulcerations; swelling, thickening and detachment of villi; no changes in goblet cells; upregulation of IL1β, IL8 and IL10[48]
	Larva (3-8 dpf)	50-75 μg/mL TNBS in swimming water (E3 medium)	Dose-dependent survival; expansion of intestinal lumen; loss of villi; increased number of goblet cell; upregulation of IL1β, TNFα, IL8, and MMP9; increased TNFα expression in lumen; infiltrate of myeloid cells[59,60]
DSS	Larva (3-6 dpf)	0.5% DSS in swimming water (E3 medium)	Mucosecretory phenotype; neutrophilic infiltration microbiota - dependent; upregulation of CCl20, IL1β, IL23, IL8, MMP9 and PCNAIL; increased proliferating cells[76]
Glafenine	Larva (5 dpf)	25 μmol/L glafenine for 12 h in in swimming water (E3 medium)	Apoptosis in intestinal epithelial cells; ER stress in IECs[79]

TNBS: 2,4,6-trinitrobenzenesulfonic acid; DSS: Dextran sulfate sodium.

increased total triglycerides, present larger visceral adipocytes and increased linear growth, compared with wild type (WT) animals. However, though they could not demonstrate the direct action of the zebrafish AgRP protein as a competitive antagonist of the melanocortin receptors, the authors showed that the positive response to α-melanocyte stimulating hormone (α-MSH) by zebrafish MC3R, MC4R and MC5bR transfected cells could be antagonized with mouse-AgRP. Further research on this pathway revealed an *in vivo* interaction of MC4R with two forms of melanocortin receptor accessory protein 2 (MRAP2) in zebrafish[103]. In cell culture, MRAP2a binds to MC4R and reduces its ability to bind to its ligand αMSH, and *in vivo,* MRAP2a is expressed during larval stages and stimulates growth by blocking the action of MC4R. The MC4R antagonist AgRP is also highly expressed in larvae and collaborates with MARP2 to maintain MC4R in a stable inactive state. On the other hand, MRAP2b is highly expressed in the adult zebrafish brain and it causes a moderate increase in the expression of MC4R in transfected cells, and even increase MC4R affinity to its ligand αMSH, suggesting that MRAP2b is the homologous isoform of the mammalian MRAP2. Given the chronic inflammatory state in obese mammals, it would be interesting to analyze the inflammatory state resulting from genetic modifications of this pathway, in order to find a genetic relationship to inflammation.

An interesting transgenic of exogenous human constitutively active Akt1 (*myrAkt1*) expressed in the skin protein Keratin-4 (*krt4*) presented a severe obese phenotype in adult zebrafish[104]. Tg(*krt4:Hsa.myrAkt1*)^{cy18} animals exhibit hypertrophic and hyperplastic growth of the epidermis during the larval stages, caused by the upregulation of the activated Akt1 downstream targets glycogen synthase kinase 3 alpha/beta (GSK3α/β), mammalian target of rapamycin (mTOR) and 70-kDa S6 protein kinase (70S6K). The adult Tg(*krt4:Hsa.myrAkt1*)^{cy18} had an increased body weight but not body length and also an augmented conditional factor, equivalent to human BMI, compared to WT siblings. Analysis with Oil Red O revealed pronounced lipid accumulation in the entire body that primarily arose from an excess of triglycerides with normal cholesterol accumulation. Sagittal sections of the entire body of obese transgenic adult zebrafish showed adipocyte hyperplasia rather than hypertrophy, as well as ectopic adipocytes in the muscles of the dorsal body and the gill arch that also infiltrated and replaced bone and skeletal muscle cells. This seemed to be triggered by upregulation of the ectopic expression of *myAkt1* in liver, muscle and bone and activation of the mTOR pathway in adipose tissue. Exploring the mRNA expression in tail samples of Tg (*krt4:Hsa.myrAkt1*) cy18, Rasouli *et al*[105] found downregulation of the transcripts of myogenic factor 5 (*myf5*), myogenic factor 6 (*myf6*) myogenic differentiation 1 (*myod1*) and myosin light polypeptide 2 (*mylz2*), which are myogenic regulatory factors and structural proteins. Factors participating of skeletogenesis as runt-related transcription factor 2 (*runx2)* and collagen type Ⅱ alpha-1a (*col2α1a*) were also down-regulated, while genes related to lipid metabolism such as *pparγ* and CCAAT/enhancer binding protein α (*cebpα*) were intensely upregulated, in addition to fatty acid-binding proteins (*fabp11a* and *fabp11b*), sterol regulatory element binding transcription factor 1 (*srebf1*), lipoprotein lipase (*lpl*) and stearoyl-CoA desaturase (*scd*). Analysis of the inflammatory state of this transgenic animal revealed high expression of adiponectin (*adipoql* and *adipoql2*), of the adiponectin receptors *adipor1a* and *adipor1b*, the leptin receptor (*lepr*), and *lipin1*, known as adipocytokines. Inflammatory molecules such as *tnfα, il1β, mmp2* and *mmp9* were also upregulated, and although no differences in the number of whole body neutrophils were found, neutrophil aggregation could be seen in the obese animals' tails. It would be interesting to see if these neutrophils aggregate in WAT. Other characteristics of this transgenic animal were a "sedentary" swimming behavior because muscles were replaced by fat, a lower survival rate compared with WT, and reduced glucose clearance after feeding, suggesting impaired glucose tolerance in these animals.

Diet induced obesity models

In addition to the genetic mutations that could lead to

an obesogenic phenotype several diet induced obesity (DIO) models also exist, that use different combinations of high fat food to generate the phenotype. Oka *et al*[106] designed a DIO model in zebrafish by overfeeding adults for 8 wk with freshly hatched nauplii *Artemia* (brine shrimp), which are part of the normal food in zebrafish facilities. The DIO animals exhibited an increased BMI (calculated as the body weight divided by the square of the body length), increased plasma triglycerides and hepatosteatosis. These parameters were improved by a calorie restricted diet following overfeeding. A comparative transcriptome analysis between visceral adipose tissue of DIO zebrafish, DIO mice, DIO rats and obese humans revealed common pathophysiological pathways. Genes related to blood coagulation and lipid metabolism were significantly dysregulated in the four obese groups, including apolipoprotein H (*apoh*), *il6* and *il1β* as regulatory molecules appearing in the coagulation cascade, as well as *srebf1*, peroxisome proliferator activated receptor alpha (*pparα*) and gamma (*pparγ),* nuclear receptor subfamily 1 group H member 3 (*nr1h3*) and leptin (*lep*), which are regulatory molecules involved in lipid metabolism in obese zebrafish, rats, mice and humans. These results indicates that immune molecules occur in obesity pathways in both zebrafish and mammals. Further research with this DIO model tested the anti-obesity effects of different vegetables in zebrafish, including regular and Campari tomatoes, pumpkins, eggplants, and others[107]. Campari tomatoes have significant lipid-lowering proprieties because they suppressed the increase of body weight and plasma triglycerides in DIO zebrafish, reduced lipid accumulation in the liver, and increased the genes involved in fatty acid oxidation such as proliferator-activated receptor gamma co-activator 1α (*pparγc1α*) and peroxisome proliferator-activated receptor αb (*ppar-αb*). Additionally, the same group tested the anti-adipogenesis proprieties of green tea extract (GTE) in the same DIO model[108]. GTE treatment decreased the volume of visceral but not subcutaneous WAT and increased the liver expression of acyl-coenzyme A oxidase 1, palmitoyl (*acox1*), acyl-coenzyme A dehydrogenase (*acadam*) and *pparα*, which are part of β-oxidation and lipid catabolism. Also decreased the expression of suppressor of cytokine signaling 3b (*socs3*) in visceral fat, which inhibits leptin signaling. Another approach using GTE as a treatment in adult zebrafish used a standard chow supplemented with gluten, α-potato starch, corn oil and lard in order to create four diets for DIO models with different fat contents. The results indicated no differences in fat accumulation between the groups of high-fat (HF) or of low-fat (LF). As in the previous model, GTE decreased body weight and fat volume in animals on a HF diet, and increased the activity of the enzyme 3-hydroxyacyl-coenzyme A dehydrogenase in liver and skeletal muscle, which is part of the β-oxidation pathway, demonstrating the utility of this model to test different natural anti-obesogenic compounds.

Another recent DIO model based on overfeeding adult zebrafish was generated by feeding them two times the standard fish chow than the controls[109]. DIO animals showed increased total weight, showed liver steatosis, as well as the overexpression of *tac4*, *col4a3*, *col4a5*, lysyl oxidases and genes involved in retinoid metabolism. A liver transcriptomic analysis after inoculation with LPS showed that immune system genes responded to LPS stimulation, including Toll-like receptors, ubiquitin mediated proteolysis, RIG-I-like receptor signaling pathway, MAPK and Jak–STAT signaling pathway in control lean animals. No alterations were observed in obese animals, and there was also no difference between obese animals and uninjected obese controls. Studying the differences between obese and non-obese zebrafish in other organs during LPS-stimulation or in other infection models could be of great interest, because obese animals are in a basal inflammatory state.

Though adult models of obesity seems to be more popular, larvae obesity models are also promising. The zebrafish obesogenic test[110] was created for the *in vivo* study of the effect of diet composition, chemical pollutants, and/or drugs on white adipocyte tissue. Larvae of a standard length of 7.5-9 mm were fed with a three-day protocol, in which the first day started with a high-fat diet (HFD) based on hard-boiled chicken egg-yolks *ad libitum* for the entire day, followed by starvation the next day, and by exposure to different obesogenic/non-obesogenic compounds the third day. After the feeding period, HFD animals showed an increase in NR staining in blood vessels, which were reduced after the fasting period. This phenomenon was not observed in control animals fed with a standard diet (SD). When studying the interaction of the initial diet with different compounds, the researchers found that exposure to rosiglitazone, a PPARγ agonist used in type Ⅱ diabetes treatment, increased the lipids deposits in both SD and HFD animals, and this effect was inhibited by T0070907, a PPARγ antagonist. A similar result was observed with tributyltin, a renowned environmental obesogen that binds to PPARγ and retinoid X receptor, for which both SD and HDF larvae exhibited an increase in adipose deposits. However, additive effects between any of the two chemical and HFD were not observed. Although this model showed an increase in blood vessel lipids for a HFD in a short period of time, this result does not reflect obesity as a chronic disease, because differences in the accumulation of lipids in the visceral WAT between SD and HFD animals or increase in weight and size of HFD larvae were not observed. Perhaps a longer exposure period to the HFD would affect these parameters.

Progatzky *et al*[111], showed that the exposure to a HF diet or a high-cholesterol diet (HCD) in zebrafish larvae induced an inflammatory response in hours, with infiltration of myeloid cells in the intestine, dependent on inflammasome activation by IECs. They demonstrated that the inflammation was directly induced by cholesterol binding to the Niemann-Pick C1-like receptor, with the participation of the apoptosis-associated speck-like protein containing a CARD (ASC) and activation of

Table 2 Zebrafish models of obesity

Model	Age	Induction	Characteristics
Genetic models			
AgRP overexpression	All stages	AgRP expressed under the control of β-actin promoter	Weight gain and linear growth; increased BMI; visceral adipose accumulation; increased triglycerides; larger visceral adipocytes[102]
Tg(krt4:Hsa.myrAkt1)cy18	All stages	Expression of constitutively active human AKt1	Weight gain; increased BMI; triglycerides accumulation; adipocyte hyperplasia; ectopic adipose tissue; increased expression of adiponectin, adiponectin receptors, leptin receptor; increased inflammatory molecules TNFα, IL1β, MMP2 and MMP9[104]
DIO models			
Artemia overfeeding	Adult	Overfeeding with nauplii artemia for 8 wk	Increased BMI; high plasma triglycerides; hepatosteatosis[106]
Chow overfeeding	Adult	Overfeeding with standard fish chow for 8 mo	Weight gain; hepatosteatosis[109]
Zebrafish obesogenic test (OZ)	Larva	High-fat diet based in hard-boiled chicken egg-yolk ad libitum during one day	Increase in blood vessel lipids in a short time[110]
HCD	Larva	HCD, cholesterol mixed in fish standard dry food for 6 h. Extended HCD for 10 d	Infiltration of myeloid cells in intestine dependent of the inflammasome, microbiota and NFκB activation; extended feeding leads to visceral fat accumulation, liver steatosis, intestine inflammation, impaired peristalsis[111]

HCD: High cholesterol diet; BMI: Body mass index; TNF: Tumor necrosis factor; IL: Interleukin; MMP: Matrix metalloprotease.

caspase-1, which is part of the inflammasome complex[112] that produces high levels of active IL-1β. Furthermore, this inflammation was dependent on the microbiota and NFκB activation. Finally, extended feeding with a HCD produced the accumulation of visceral fat, liver steatosis, sustained inflammation in the intestine, and impaired peristalsis. This study verified a direct link between inflammation and high-fat diets, specifically the activation of the inflammasome complex by cholesterol in the intestine, and opened a new window to the study of innate inflammation in the context of obesity and its influence in other chronic inflammatory diseases.

By last, a study analyzing two flame retardants, tetrabromobisphenol-A and tetrachlorobisphenol-A, as possible obesogens using zebrafish larvae[113] showed lipid accumulation in larval stage and late-onset weight gain in juvenile animals, which was most likely caused by the compounds' activity as a PPARγ agonist. This method could be interesting for the analysis of the inflammatory state under such conditions, using these substances as agonists of PPARγ.

The zebrafish models related to obesity maybe are not so well known as mice models, nonetheless, the examples presented here (Table 2) are evidence of the conserved signals that control lipids metabolism and the flexibility of the zebrafish as model of metabolic diseases.

CONCLUSION

The models presented in this review exhibit the utility of zebrafish as a model of diseases and demonstrate that this animal as an intermediate between models involving simpler invertebrates and more complex higher mammals and can be used as an alternative or a complement to pre-clinical and drug screening studies that involve conserved metabolic and inflammatory pathways. Furthermore, the characteristics of zebrafish such as physiological homology, rapid development and a low cost of production, make this animal a great option for research on new therapies for inflammatory diseases.

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REVIEW

Hepatocellular carcinoma: Where are we?

Roberto Mazzanti, Umberto Arena, Renato Tassi

Roberto Mazzanti, Umberto Arena, Renato Tassi, Department of Experimental and Clinical Medicine, School of Sciences of Human Health, University of Florence, 50134 Firenze, Italy

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Correspondence to: Roberto Mazzanti, MD, Professor, Department of Experimental and Clinical Medicine, School of Sciences of Human Health, University of Florence, Largo Brambilla 3, 50134 Firenze, Italy. roberto.mazzanti@unifi.it
Telephone: +39-055-416635
Fax: +39-055-417123

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Abstract

Hepatocellular carcinoma (HCC) is the second cause of death due to malignancy in the world, following lung cancer. The geographic distribution of this disease accompanies its principal risk factors: Chronic hepatitis B virus and hepatitis C virus infection, alcoholism, aflatoxin B1 intoxication, liver cirrhosis, and some genetic attributes. Recently, type Ⅱ diabetes has been shown to be a risk factor for HCC together with obesity and metabolic syndrome. Although the risk factors are quite well known and it is possible to diagnose HCC when the tumor is less than 1 cm diameter, it remains elusive at the beginning and treatment is often unsuccessful. Liver transplantation is thus far considered the best treatment for HCC as it cures HCC and the underlying liver disease. Using the Milan criteria, overall survival after liver transplantation for HCC is about 70% after 5 years. Many attempts have been made to go beyond the Milan Criteria and according to recent works reasonably good results have been achieved by using a histochemical marker such as cytokeratine 19 and the so-called "up to seven criteria" to divide patients into categories according to their risk of relapse. In addition to liver transplantation other therapies have been proposed such as resection, tumor ablation by different means, embolization and chemotherapy. An important step in the treatment of advanced HCC has been the introduction of sorafenib, the first oral, systemic drug that has provided significant improvement in survival. Treatment of HCC patients must be multidisciplinary and by using the different approaches discussed in this review it is possible to offer prolonged survival and quite good and sometimes even excellent quality of life to many patients.

Key words: Hepatocellular carcinoma; Treatment; Liver cancer; Epidemiology; Liver transplantation; Percutaneous ethanol injection; Chemoembolization; Chemotherapy; Radiofrequency ablation

Core tip: This review summarizes on the current state of the art of treatment of hepatocellular carcinoma (HCC). After a brief chapter on epidemiology, risk factors and biology of HCC, the review presents all possible therapeutic approaches for HCC, from the most effective

such as liver transplantation to important but palliative treatments which can prolong patient survival such as different types of trans-artery-chemo-embolization and chemotherapy.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the ten most commonly occurring solid cancers worldwide and is the second cause of death from malignancy. The most recent data indicate that its incidence is still increasing in many countries whereas the most effective way of reducing mortality due to HCC is prevention[1].

These observations appear to be in contrast with what we know about this lethal cancer. Most of its risk factors are known. It is possible to diagnose HCC when the tumor is 1 cm diameter or less and it can be treated either surgically or medically. So why we are losing the battle against an enemy we know so well?

It is clear that there are some other risk factors which are not yet known. We do not understand the precise mechanisms by which liver cells become neoplastic clones without the possibility of our immune system to controlling them. Most important of all, the natural history of HCC before it has been diagnosed is unknown. Sophisticated technology for searching for cancer cells in blood has revealed that even small tumors distribute thousands of cancer cells into the circulation that then may begin the process of metastasis much earlier than was formerly thought[2]. In other words, the concept of "early cancer" that has been applied successfully to other situations, such as gastric cancer, has not yet proven for HCC. Although it is believed that not all viable cancer cells are able to metastasize, but only those with stem cells properties, we must consider these aspects before saying we can cure cancer patients[2,3]. Relapse can occur years later, well after the standard 5 years that are considered. Breast cancer is the best example of this phenomenon.

Liver cells undergo replicative activity throughout life (thus the concept of the "streaming liver") but it has been not clarified whether this process helps to eliminate "initiated" cells or, on the contrary, helps them to arrive in the blood circulation. Moreover, since HCC occurs mostly in the cirrhotic or chronically inflamed liver, it is possible that chronic liver disease encourages the process of metastasis from tiny neoplastic nodules.

This review will focus on some of these aspects of HCC and the various current and future treatment options.

EPIDEMIOLOGY

According to the most recent data, the global incidence of HCC is still increasing, although it varies throughout the world. The highest rates occur in Eastern Asia whereas the positive effect of vaccination against hepatitis B virus (HBV) infection is showing the first results in China and Taiwan now. The lowest rates are in Northern Europe and North America. These data are mainly explained on the basis that two of the risk factors for HCC, HBV and hepatitis C virus (HCV) infections, are largely diffused in Eastern Asia. In Italy, for example, HBV-related HCC is now rapidly declining, in comparison to HCV-related HCC because of the introduction of mass HBV vaccination several years ago[4,5]. Moreover, aflatoxin-B1 intoxication, an important risk factor for HCC, is quite common in those geographic areas where the incidence of HCC is high due to inadequate cereals and food storage conditions. The known risk factors for HCC are reported in Table 1.

Some pathological aspects of the liver such as cirrhosis and fibrosis, conditions resulting from chronic inflammation due to chronic HBV and HCV infection or to alcohol intoxication, can lead to cancer through several mechanisms, some of which are linked to chronic inflammation such as hepatocyte necrosis, liver regeneration and fibrosis and others that are specific of each virus. For example, HBV causes about 70%-80% of HCC cases in East Asia, and possesses transformation activity due to some of its proteins, *i.e.*, protein X, or by integrating part of its DNA genome into that of humans and thus altering it[6,7]. HCV, which is an RNA virus without an inverse transcriptase, cannot integrate its genome with the human, but it has several proteins that have transforming properties. In addition, HCV infects the immune system and endothelial cells, leading to immune dysfunction and chronic deregulated angiogenesis. Both conditions are known risk factors for cancer[8,9].

Alcoholism is an important risk factor for HCC because it causes fatty liver, necro-inflammation, fibrosis, liver cirrhosis and malnutrition, especially when it is associated with HCV infection. Alcohol abuse is the most important risk factor for HCC in North America and Northern Europe.

There are other risk factors for HCC, *i.e.*, hemochromatosis, fructosemia, tyrosinemia, galactosidemia, diabetes type Ⅱ, genetic propensity, or clinical conditions such as obesity or hypothyroidism. Hemochromatosis, for instance, is the most common genetic defect (1/200 born in the West) and it leads to the accumulation of iron in the body, causing heart, joint, endocrine and liver diseases. Its prevention could be a key step to preventing HCC and other fatal diseases.

In recent years, other important risk factors have been described such as type Ⅱ diabetes, metabolic syndrome and non-alcoholic steato-hepatitis. Type Ⅱ diabetes is so common in western countries (about 10% of the adult population) that it constitutes an enormous societal health problem. Type Ⅱ diabetes also increases the risk for other types of cancer, especially the most deadly pancreatic cancer. It is also the main risk factor for heart and vessel diseases. Since its occurrence is much facilitated by obesity and the lack of physical exercise,

Table 1 Identified risk factors for hepatocellular carcinoma

Age
Ethnicity
Male Gender
Liver cirrhosis
Chronic HBV infection
Chronic HCV infection
Hemochromatosis
Alcoholism
Aflatoxin-B1 intoxication
Tyrosinemia, galactosidemia, fructosemia
Alpha1 anti-trypsin deficiency
Genetics predisposition
Anabolizing hormones
Estrogen contraceptives
Obesity
Type Ⅱ diabetes
Glucose overload
Metabolic syndrome
Hypothyroidism
Fatty liver
Non-alcoholic steato-hepatitis

HBV: Hepatitis B virus; HCV: Hepatitis C virus.

its prevention should become a target for all healthcare systems in developed countries where large portions of the population are becoming more and more overweight and sedentary.

As mentioned above, also fatty liver significantly increases the risk of HCC[10]. This condition per se does not seem a risk factor, but when it becomes the expression of underlying metabolic disorders or other more severe diseases it may become a predisposing lesion of HCC, as in the large use of anabolic hormones to increase body mass. On the other hand, it now seems possible to exclude the use of modern contraceptive pills from this category of risk factors[11]. Estrogen actually may reduce the risk of HCC as suggested by the observation that HCC occurs more frequently in men rather women with a ratio ranging from 2:1 to 4:1.

Ionizing radiation and smoking are also risk factors for HCC although there are no specific works on these. Finally, last but not least, we cannot forget that HCC is an age-related disease. Although it can occur at every age from infants to the elderly, nevertheless its frequency increases in individuals from 30 to 70 years of age, according to the population under consideration (in much younger individuals in Africa and East Asia where HBV is prevalent, and in older subjects in Europe or America).

The process that transforms a normal liver cell into neoplastic one is not known, although changes in normal liver histology are likely to play an important role together with genetic alterations due to viruses, oxygen derived free radicals, toxic compounds and changes in the host immune system.

Cancer is a genetic- and age-related disease and HCC is not an exception. All the risk factors that we have taken into consideration may result in changes in the hepatocyte DNA and, since the process of transformation is quite long, the immune system is also involved in the development of clinically evident cancer. This means that the pathway to HCC must be long enough to cause an irreversible phenomenon. Chronic liver diseases and cirrhosis are very good examples of this old observation. There are thousands of patients who have had acute HBV or HCV infections many years earlier and then who developed HCC after a chronic disease that lasted decades in the literature. If either HBV or HCV are directly and rapidly oncogenic as experimental research shows, why does it take so long to develop HCC in humans? We know that HBV-related protein X or HCV NS3, NS5 or core proteins or others have the capacity to transform normal cells *in vitro*, but why does this not happen in all patients and, more importantly, why does it take 20-30 years for a neoplastic nodule to appear in man? Is it only due to our still rudimentary diagnostic tools which do not perceive tiny cancerous foci, or are some individual's immune systems able to combat the disease, or are there more complex pathways which we do not yet understand?

Consider the recent data published by two of the top scientists worldwide on colon cancer: The main risk factor according to their data is bad luck. After thousands of papers published on this issue, billions of dollars spent on research on it, the bitter conclusion is that the most important factor, but not the only one, in getting colon or lung cancer is bad luck[12]. What a terrible conclusion!

The liver is an example that fits quite well what Tomasetti and Vogelstein have shown[12]. Since liver stem cells have quite intense replicative activity and since the greater the replication, the higher the risk that one or more daughter cells have mutation in their DNA, it makes sense that the risk of acquiring HCC is higher in the presence of risk factors, such as HBV or HCV infection, alcoholism, aging, excessive iron, ionizing radiation, and smoking, however HCC also occurs in subjects who do not have any of the above risk factors.

Vaccination against HBV infection, prevention of HCV-infected transfusions, prevention of alcoholism or aflatoxin intoxication are crucial factors in the prevention of HCC. And we should also mention that besides the risk factors, there are also protective conditions that could help us to understand better how cancer develops. The most important protective factor for HCC is vaccination against HBV infection. Prevention of chronic HBV carrier status and chronic liver disease is crucial as shown recently by the decrease in HCC in Taiwan and China after the adoption of mass vaccination[4]. Reducing the HBV load in patients with liver cirrhosis and chronic hepatitis reduces also reduces the risk of HCC[13]. There are other factors that can help protect from HCC, such as regular consumption of vegetables and coffee drinking (3-4 cups/d). Vitamin D and calcium intake may also exert a protective role although results are controversial[14-17] (Table 2). It is also possible that other dietary factors act as protective factors, including consumption of phenols contained in red wine, green tea, olive oil, but these data

Table 2 Protective or probably protective factors

HBV vaccination
Physical exercise
Balanced diet
High vegetable consumption
Calcium
Vitamin D
Coffee

HBV: Hepatitis B virus.

require confirmation[18].

BIOLOGICAL FEATURES OF HCC

Despite recent advances in surveillance and management of HCC, its molecular biology is quite elusive. A complete definition of molecular events that lead to hepato-carcinogenesis is a major challenge in the clinical management of this disease. There is no doubt, however, that liver cirrhosis, chronic hepatitis, chronic HBV and HCV infections, and intoxication by alcohol, iron and aflatoxin-B1 all play a major role in causing HCC.

Hepato-carcinogenesis is a multi-step process that begins from an altered hepatic microenvironment, typically related to chronic liver disease, characterized by massive inflammation and fibrosis that are responsible for the deregulation of several signaling pathways and accumulation of genetic alterations in normal hepatocytes[19-21]. The latter are finally transformed into dysplastic lesions causing early carcinoma which finally progresses to HCC[22]. What seems particularly important in liver oncogenesis in humans is the lag time from the beginning of the inflammatory process or the exposure to risk factors and the occurrence of HCC. This review does not address specific aspects that could be involved in liver oncogenesis, but we do present some updated references that can provide more thorough information to the interested reader.

HCC is an extremely heterogeneous solid cancer[23] and factors such as platelet derived growth factor, transforming growth factors (TGFs), vascular endothelial growth factor (VEGF), hepatocyte growth factor, reactive oxygen species (ROS) and cellular factors such as hepatocytes, stem like, stellate and inflammatory cells are involved[24,25]. We believe that ROS and the oxidative stress that are associated with inflammation are of particular importance[26]. HCV infection, alcohol abuse or iron intoxication, for instance, all cause ROS and reactive nitrogen species (RNS) that can eventually overcome the liver's defense against oxidative stress, thus damaging DNA and cell proteins[27]. HBV-related inflammation also causes oxidative stress due to both the inflammation itself and protein X that stresses the endoplasmic reticulum *via* cAMP-responsive element-binding protein (CREBH)[26]. The presence of transforming proteins such as HBVx protein or HCV-related NS3, NS5, or core proteins may all lead to hepatocyte transformation. Intense angiogenesis in the liver during chronic HCV infection is also important and thus these patients are at greater risk of developing HCC[28,29].

Understanding the precise molecular processes occurring during these steps may offer a better therapeutic approach to HCC. Unfortunately, our understanding of how things occur *in vivo* is not sufficient to drive safe and effective therapy, which is the main reason why traditional chemo- and hormone therapies have produced discouraging results[30]. From a molecular point of view HCC is a heterogeneous disease that shows heterogeneity between different tumor nodules in the same patient and differences in the same nodule itself[31,32]. This may reflect the existence of distinct pools of cancer stem-like cells with different oncogenicity and independent genomic evolution. Thus, not only does each patient have his/her own HCC, but each nodule may be genetically unique[32-35]. With regard to this point, unfortunately the theory about the importance of cancer stem-like cells in HCC has produced little. There is a controversy regarding what they are and where they come from in HCC as well as in other cancers[36-39]. Like many, we think that they come from oval cells that, after prolonged proliferative activity, evolve towards a sort of erroneous differentiation. It is possible that oxidative stress causes DNA damage that results in initiation of the process. However, this is pure speculation, as we simply do not know! What we know very well is that without liver cirrhosis, chronic HBV and HCV infections or iron, ethanol or aflatoxin B1 intoxication, HCC is a rare disease.

MOLECULAR ASPECTS OF HCC

Genome-wide surveys have been used to try to better understand HCC molecular mechanisms. Pathways commonly altered by genetic alterations (somatic mutations or homozygous deletions) include the Wnt/beta-catenin pathway, the p53 pathway, phosphatidylinositol 3-kinase (PI3K)/Ras signaling pathways, oxidative and endoplasmic reticulum stress modulators and processes responsible for chromatin remodeling[40]. Interestingly, different mutations segregated by the etiology of the underlying liver disease suggest that the carcinogenesis and development of mutations causing cancer may vary, depending on pre-existing liver disease. For example, inactivation of genes involved in chromatin remodeling is more common in patients with alcoholic cirrhosis, while p53 pathway deregulation is predominant in HBV-positive patients and Ras in those affected by HCV[40-43].

Similar results have recently been obtained through transcriptome analysis of liver cancer that has shown a deregulation in some oncogenic signaling pathways such as those of TGF-β1, MYC, PI3K/Akt, Wnt/β-catenin, NOTCH and MET[41-43].

Some of these HCC hallmarks have been analyzed in depth and their role as a potential target for HCC treatment is under investigation. VEGF and its receptors are known to be key mediators in HCC initiation, growth and diffusion[44,45]. Increased VEGF expression from low to high grade dysplastic nodules or advanced HCC and the correlation of VEGF levels with tumor stage and risk

of recurrence, support this hypothesis[44-46]. We have confirmed the importance of angiogenic phenotype in HCV-related HCC[47]. Fibroblast growth factor (FGF) and its family of receptors are recognized as a central player in augmenting HCC growth, invasion, and angiogenesis, making it an intriguing candidate for HCC therapy. FGF activation is involved in neovascularization[48]. Increased serum levels of FGF or activation of its receptors are associated with recurrence, treatment resistance, and poor prognosis[49] while synergistic interactions between FGF and VEGF have been shown to be a resistance mechanism to the antiangiogenic effects of VEGF-targeted agents[50].

Several other pathways have been shown to be involved in HCC pathogenesis. In particular, promising results come from the PI3K/Akt/mTOR, EGFR, Ras/Raf/mitogen-activated protein kinase (MEK)/extracellular-signal-regulated kinase (ERK), and IGFR pathways[51,52].

The PI3K/Akt/mTOR pathway is a major intracellular signaling cascade that is involved in the regulation of cell proliferation, growth and survival, through activation of tyrosine kinase receptors, such as VEGFR, EGFR, PDGFR, and IGFR. Nearly 50% of patients with HCC exhibit activation of the mTOR pathway, which may be partially attributable to activation signals from receptor tyrosine kinases such as IGFR and/or EGFR pathways. mTOR pathway activation is associated with aggressive HCC and decreased survival in these patients[53]. Despite promising results targeting mTOR, a substantial benefit in HCC patients has not been shown in clinical practice.

The Ras/Raf/MEK/ERK signaling cascade is another important intracellular pathway altered in HCC such as the Wnt pathway, however, while Raf inhibitors such as sorafenib have been developed in HCC therapy, Wnt inhibitors have not yet been developed.

cMET, a tyrosine kinase receptor with its ligand hepatocyte growth factor, has been implicated in HCC and probably in multidrug resistance[54] and represents a target that is undergoing intense investigation. Randomized phase Ⅱ data of the cMET inhibitor, tivantinib, has demonstrated activity in patients with advanced HCC who progress on sorafenib with elevated expression of cMET by immunohistochemistry. Tivantinib is undergoing phase Ⅲ investigation in this subgroup of patients[55,56].

Given the molecular heterogeneity of HCC, the challenge is to identify in which patients any given alteration is critical. It is unlikely that any of these targeted agents will yield clinical success without selecting the patients whose tumors are most dependent on these pathways and are therefore most likely to benefit, and the identification of predictive markers of response is essential for the successful development of new targeted agents.

SURVEILLANCE PROGRAMS AND DIAGNOSIS

If we exclude the so-called "incidental" occurrence of HCC that is found by chance in subjects undergoing ultrasound scan and/or computerized tomography (CT) for other reasons, most HCC cases are suspected and diagnosed in a program of clinical controls or planned controls in patients known to be at risk for the disease. It is possible to diagnose HCC using different imaging techniques such as ultrasound, CT, nuclear magnetic resonance (NMR), but confirmation comes only with histology. There is a general agreement now that ultrasound should be the first tool for detecting HCC nodules in patients admitted to follow-up because of chronic liver disease or for the existence of an at-risk condition. Blood tests are much less useful, and they may help more in patient follow-up rather than in diagnosis. It is possible to find diagnostic algorithms made by experts after extensive discussion in the international literature[57,58]. However, we believe that confirmation of the presence of HCC by histology should be searched in all cases where image means (CT and/or NMR) are not conclusive or legal controversy may arise when such confirmation is lacking.

As mentioned above, HCC can first be observed by ultrasound scan, which is an inexpensive, easy, fast, and readily available technology in every part of the globe. Most scientific societies for the study of liver diseases agree that a 6-mo interval is the most convenient for checking up the development of HCC in liver patients[57,58] (Table 3).

Some authors suggest that surveillance becomes convenient when the ratio between cost and benefit achieves 3 extra mo of life at a cost of less than 50000 dollars[59]. The 6-mo interval comes from a large number of studies that have shown that the doubling time of HCC is about 6-mo, although very few of them have shown a clear gain in survival as compared to groups of patients who did not undergo programmed check-up[60].

The effectiveness of ultrasound in detecting any form of HCC is quite good (94%), but its sensitivity drops to 63% when the target to be detected is small[61,62]. Because no ethical committee would permit a comparative study between controlled and uncontrolled cirrhotic patients, we think that the 6-mo interval is reasonable to follow-up patients. This interval becomes useless for assessing the response to treatment or to check for relapse in patients who have been treated for HCC. In addition, in patients where ultrasound has shown lesions of less than 1 cm diameter it should be repeated with a much shorter interval than 6-mo (we use 3-mo, normally). It is clear that once liver patients have been treated for HCC, they should be carefully followed-up with intervals inferior to 6-mo, either to follow how the treated nodule(s) is doing or to assess new lesions.

There is another exception that must be considered and it concerns those who are on the waiting list for liver transplantation. The occurrence of a small nodule of suspected HCC puts these patients into the priority line for transplantation according to the Milan criteria[61,62].

Contrast-enhanced ultrasound does not increase the sensitivity for small HCC, but it helps in differentiating some lesions[63]. It is common that most of those with liver nodules undergo ultrasound-guided needle biopsy to assess the histology of the lesion. Expert pathologists

Table 3 Patients where surveillance program is suggested by International Societies for the Study of the Liver (AASLD, EASL-EORTC, JSH, APASL)

Society	Target population	Target population
APASL, 2010	Chronic hepatitis	Cirrhosis
	No recommendation	HBV and HCV cirrhosis
AASLD, 2011	HBV-positive patients	HBV and/or HCV cirrhotics
	Male Asian HBV+ > 40 yr	Fourth stage PBC
	African > 20 yr; Familial predisposition + for HCC	a1-antitrypsin deficit
		Autoimmune hepatitis
		NASH
JSH, 2011	HBV and HCV chronic hepatitis	Non viral cirrhosis
		HBV and HCV cirrhosis
EASL, 2012	HBV + active hepatitis	Child A and B cirrhosis
	HCV + hepatitis with advanced fibrosis F3 (Metavir)	Child C cirrhosis with indication to liver transplantation

AASLD: American Association for the Study of Liver Diseases; JSH: Japan Society of Hepatology; EASL: European Association for the Study of the Liver; APASL: Asian Pacific Association for the Study of the Liver; NASH: Non-alcoholic steato-hepatitis; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; PBC: Primary biliary cirrhosis; EORTC: European Organization for Research and Treatment of Cancer.

are needed to solve sometimes difficult diagnoses and the use of ultrasound scan, CT, or even NMR are very helpful in some cases. Before biopsy there are other techniques that help clinicians to reach the correct diagnosis.

Radiologists have studied the behavior of blood distribution inside the tumor, differentiating the artery phase from the portal phase (early and tardive). By using liver specific contrast it is possible to observe how the liver handles the contrast, suggesting the presence of HCC tissue or benign lesions. HCC is commonly a hypervascular lesion and contrasted CT can distinguish a rapid wash–in phase of the tumor (artery supply) from liver tissue that surrounds the HCC nodule. In patients with chronic liver disease the occurrence of a lesion having such characteristics strongly suggests the presence of HCC independently from other signs. Sometimes lesions are hypo-vascularized, especially those with a diameter of less than 2 cm. In these cases the most sensitive and specific technique is NMR with hepato-specific contrast that distinguishes the hypo-vascular lesion from other aspects of the liver, taking advantage of the absence of any metabolism of the contrast by neoplastic cells[64-68].

In patients with chronic liver disease, especially with liver cirrhosis, it is relatively common to observe lesions of less than 1 cm diameter by ultrasound and CT scan. These lesions are difficult to diagnose, even when using ultrasound-guided biopsy because of the occurrence of false negatives. It is more prudent to wait and watch before doing anything, unless the patient is on the waiting list for liver transplantation. As mentioned before, this occurrence can move the patient into priority line for liver transplant. In patients with Child-Pugh stage A liver cirrhosis, repeated ultrasound check-ups must be done and if the lesion tends to grow, it may be considered for needle biopsy by expert hands. Once liver histology has been obtained, the pathologist should perform many specific tests such as CD 34, CK7, glypican 3, HSP-70 and others to assess the malignancy of the tissue according to guidelines of the European Association for the Study of the Liver, or the European Organization for Research and Treatment of Cancer. There is controversy among different scientific societies regarding use of contrasted ultrasound in differentiating HCC, though CEUS can better identify a small lesion in a cirrhotic liver.

An issue that has been discussed intensely concerns whether liver biopsy of suspected nodules should be performed. From what was said above, we are in favor of liver biopsy in cases where not invasive techniques are not conclusive[69]. In addition, now that target therapy is becoming a reality, we think that good quality molecular study of each biopsy could add the knowledge that is required for administering the right target therapy. Circulating cancer cells could be the alternative in the future, but at present they do not give certainty about the presence and the site of HCC[70,71].

As regards the use of biochemical blood parameters such as alpha-fetoprotein, or fucosylate alpha-pheto-protein or des-gamma-prothrombin, we agree with most western researchers that these parameters have limited utility in diagnosing HCC. They are especially misleading in screening programs because of poor sensitivity and specificity. On the contrary, they can be useful once HCC has been diagnosed to follow up the results of treatment. While this manuscript was in preparation a study concerning a possible new blood indicator for the presence of HCC in cirrhotic patients was published[72]. Only time will tell whether this new indicator for the presence of HCC will become of general use or will be disappointing, like many others.

CLINICAL ASPECTS

As most solid cancers, HCC is asymptomatic for several months and sometimes for years. This means that HCC should be searched for in all known chronic liver disease patients with adequate diagnostic tools at regular intervals.

When HCC occurs in patients with an apparently healthy liver, the diagnosis is usually late and the patient

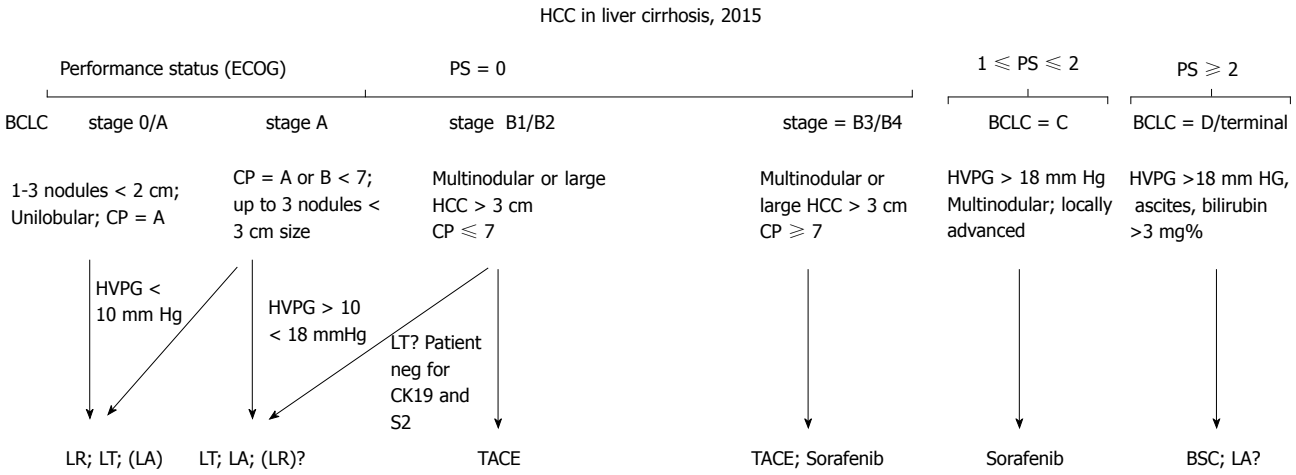

Figure 1 Staging and possible strategy to treat patients with hcc and liver cirrhosis. PS: Performance status according to ECOG; BCLC: Barcelona clinic liver cancer; HVPG: Hepatic vein portal gradient; BSC: Best supportive care; CP: Child-Pugh score; CK19: Cytokeratin 19; S2: Patients beyond Milan Criteria (see text). References[73-75,79,123].

has advanced disease.

Symptoms, when present, are those of chronic hepatitis with the addition of pain in the right superior quarter of the abdomen, increased volume, and more fatigue. If a liver disease patient presents with a change in mood, edema in the legs, swollen abdomen due to ascites, rapid occurrence or deterioration of portal hypertension with esophageal or rectal varices, hemorrhoids or bleeding, the physician should consider that HCC may be developing. Decompensation of diabetes can also be a sign of the presence of HCC. Ultrasound scan without and with liver-specific contrast should be done rapidly and followed by a CT scan with contrast medium.

Early detection of liver nodules and diagnosis of HCC is crucial to ensuring that the patient have the greatest possibility of being cured or, at least, of prolonging survival and improving quality of life. It must be remembered that only 15%-35% of those who develop HCC can undergo surgery.

A patient's prognosis strongly depends upon the condition of his/her liver at diagnosis. Because of this, several authors have tried to make a "road map" to assist in selecting the proper therapy for the right patient.

STAGING

As in all patients with cancer, staging is crucial before deciding any treatment. In addition, since more than 90% of HCC patients have an important underlying liver disease, careful staging of it must also be performed to avoid cancer treatment that damages the liver reserve. In the last 50 years several systems have been proposed to stage liver disease with or without HCC. With others we have developed in Italy a system to estimate the prognosis of patients affected by HCC and liver cirrhosis, the Cancer of the Liver Italian Program that, according to our own results and in the opinion of many other researchers has been very precise in defining the prognosis of patients[73]. However, the Barcelona Clinic Liver Classification (BCLC) system with few modifications is more frequently used now because it also takes into consideration the choice of treatment. The BCLC staging system divides HCC associated with liver cirrhosis into: Very early stage (0 stage), early stage (stage A), intermediate stage (stage B), advanced stage (stage C) and terminal stage (stage D) and, compared to others, has the advantage of taking into account not only tumor burden, stage of liver cirrhosis, liver function, physical condition but also the efficacy of available treatment[74]. Patients have the best treatment opportunities at Centers with vast experience who utilize the BCLC system, which has recently been updated because of stage B heterogeneity, so B stage can now be divided into four subgroups, B1, B2, B3, B4 according to Child Pugh, Milan criteria, number and size of lesions, and ECOG performance status (Figure 1)[75,76].

TREATMENT

The treatment of HCC is difficult and requires a multidisciplinary approach, whereby specialists in gastroenterology, hepatology, radiology, oncology, surgery and others need to bring their expertise to provide patients with the best and most updated therapies. It is possible to differentiate treatments into two main categories: Those that are potentially curative and those that are palliative.

POTENTIALLY CURATIVE TREATMENTS

In this category we consider three types of approach: Liver transplantation, liver resection, and liver ablation. Each of these approaches has indications and contraindications, and need to be discussed carefully and tailored to each individual patient.

Liver transplantation is widely considered the only real potentially curative approach that provides treatment

of both HCC and the underlying liver cirrhosis. Its gold standard indication is early unresectable HCC in patients with compensated liver cirrhosis or HCC with no more than 3 nodules, Stage 0 or A according to the BCLC system. Recent reports show that 70% of transplanted patients for HCC are alive after 5 years, especially when transplantation was performed using the Milan criteria[77-79]. This percentage of success is extremely good when considering that most of these patients had liver cirrhosis and HCC. Unfortunately, only a minority of patients can undergo liver transplantation even when it is indicated. The number of donors is largely insufficient to cope with the worldwide need and the shortage of organs makes waiting lists longer each day in every country, so that many patients become unsuitable for transplantation or die while awaiting transplant. Many attempts have been made to go beyond the limits of the Milan criteria and results are under scrutiny now. One large review involving 770 consecutive transplanted patients for HCC shows that performing transplantation beyond the Milan criteria attains a 57% general rate of survival, after 5 years, with a rate of recurrence of 35%[80]. However, by using tissue cytokeratin 19 (CK 19) and the sum of the size of the largest tumor size plus the number of nodules up to 7, as for S2 patients, as prognostic markers, it was possible to divide the entire group of patients in two: (1) one group of individuals who were negative for CK 19 and were not included in the S2 group; and (2) another group resulting positive or to be in the S2 group. The results were quite different and impressive: The first group had a 64% survival rate and 19% relapse compared to the other group where survival dropped to 45% with a rate of recurrence of 53%[80].

There are several reviews that address the issue of selection of patients to insert on the waiting list for transplantation[81,82]. In this general review it is sufficient to say that patients with early or very early unresectable HCC and liver cirrhosis with good liver reserve function (Child Pugh A) (stage 0 or A according to the BCLC system) are excellent candidates for liver transplant. Those who have poor reserve of liver function or too advanced HCC, stage C of the BCLC system, are not good candidates because of the surgery risk and/or frequent and/or rapid recurrence or appearance of metastases. Finally, there is a group of heterogeneous patients, included in the intermediate stage according to the BCLC system (stage B), where the decision on whether to submit patients to transplantation must be discussed among experts with different expertise. One possible pathway is indicated in Figure 1. Patients with Stage D disease are not candidates for transplantation nor for active treatment of HCC.

There are other problems with liver transplant: One is that many candidates for transplantation have chronic HBV or HCV infections that recur after transplantation and may quite rapidly reproduce liver cirrhosis. Effective treatments have been available for HBV infection for several years, including use of interferon, lamivudine and other antiviral drugs[83-85]. Treatment is usually effective and eradicates the infection in most. Therapy should start before surgery and be continued for several months. It is more difficult to deal with HCV infection, especially that due to HCV genotype 1 that is particularly resistant to antiviral treatment with peg-interferon and ribavirin. Relapse of chronic hepatitis and liver cirrhosis occurs rapidly and, in addition, these patients show significantly worse prognosis than others[86]. Accordingly, active HCV infection has been a reason to exclude patients from transplantation in many centers. However, major progress has been made in the last 2 years in the treatment of chronic HCV infection including that due to genotype 1, through introduction of drugs such as sofosbuvir, ledipasvir, elbasvir and others that specifically target HCV proteins[87,88]. These drugs are able to clear the virus in most HCV patients though bitter discussion is in progress in several countries over the cost of these drugs and on their generalized use. It is too early to ascertain whether these new antiviral drugs have solved the problem. It must be kept in mind that HCV infects several sanctuaries of the body and since transplanted patients are chronically treated with immunosuppressive drugs, it is not possible to exclude later relapse of infection. Moreover, reactivation of HBV infection has been reported in patients on treatment with these new anti-HCV drugs and the issue is still open to possible surprises[89].

To overcome the shortage of donors and to cut - waiting list time for recipients of a new liver it has been proposed to split the available deceased-donor livers (DDLT) or to take part of the liver from live donors (LDLT) (usually a relative of the patient). The results appear good according to what has been published especially in Asia, without any statistically significant increase in recurrence of HCC as compared to traditional cadaveric donors[90-93]. Complications of liver surgery of live donors make this option still debatable especially considering a mortality rate that ranges from 0.1% to 0.3% of the donor[93,94].

What should be done to stop HCC progression while patients are on the transplant waiting list? The ideal approach would destroy the neoplastic tissue as much as possible without damaging the rest of the liver, without reducing functional reserve and, most importantly, without increasing the number of circulating liver cancer cells that cause metastases. Radiofrequency ablation (RFA) that causes almost full coagulative necrosis of the nodule in a single shot might be the best procedure, at least for small lesions. However, the use of trans-hepatic artery embolization (TAE) or trans-hepatic artery chemo-embolization (TACE) (see later) that theoretically deprive HCC of artery blood flow, necrotizing most of the tumor while allowing the presence of other neoplastic nodules to be assessed during the same procedure, are generally used. Many papers published on this issue show that TACE provides good results and increases overall survival compared to transplantation alone or TACE alone[95]. However, there are no controlled randomized studies comparing

TACE and RFA as neoadjuvant treatment prior to liver transplant. In addition, many centers are now using a different technique of TACE that uses beads eluting anticancer drugs or ionizing microspheres charged with Yttrium-99 to embolize the artery that feeds the tumor. Results are similar in terms of overall survival, although bead-TACE seems to produce fewer side effects than traditional TACE[95-98]. Bead-TACE also appears useful in downstaging HCC or in maintaining it at a steady stage while patients are awaiting transplant[99,100].

Liver resection

Liver resection is the other surgical option for treatment of HCC[101,102]. Liver surgery in patients with cirrhosis or chronic liver disease requires expert surgeons and teams trained to work together in these patients, who may develop liver failure after large resection. In addition, recurrence of HCC within 3 years is quite common (> 50%) even after the introduction of ultrasound during surgery. Other procedures have been proposed to improve the outcome of patients with HCC and liver cirrhosis who undergo liver resection. The adoption of the Milan criteria used for liver transplantation and NMR with gadoxetic acid-enhanced MRI during surgery can improve detection of small lesions by 16% and makes surgery more precise and radical[103,104].

Liver resection has some advantages over transplantation (organ saving, costs, expertise, *etc.*) and in addition leaves open the possibility of later transplantation, thus allowing many patients to gain time. Unfortunately, liver resection does not treat the underlying cirrhosis that is a pre-neoplastic lesion and it can be too risky for patients with advanced HCC and/or liver disease (stage C according to the BCLC). Even very selective resection can cause liver decompensation and/or gastrointestinal bleeding, especially in patients with high values (>10 mmHg) of portal hypertension and gastro-esophageal varices. This limitation has been disputed recently as it excludes too many patients (about one-fourth) who could benefit from surgery from effective treatment[105].

Liver resection in patients with BCLC Stage B can be done quite safely by avoiding to operate those with serum bilirubin over 2 mg/dL and/or ascites. In selected patients, resection leads to an overall survival > 85% at 5 years, making this type of surgery the best option for treating early or very early HCC in well compensated liver cirrhosis (Child Pugh A).

Liver ablation

Liver ablation performed using different techniques can be used in patients where surgery is not possible or too risky. Ablative techniques performed by experts in selected patients can allow survival comparable to transplantation even if more recent data have questioned this conclusion[106-112]. Of the ablative techniques, Percutaneous ethanol injection (PEI) was the first to be used in patients with small lesion(s) (< 3 cm diameter) HCC. Excellent results have been observed with recurrence rates at 5 years similar to resection. PEI consists in injecting ethanol inside the tumor mass to cause coagulative necrosis of all neoplastic nodules. PEI has been the most successful technique in treating small (less than 3 cm diameter) HCC nodules. It is easy, inexpensive, requires minimal equipment and expertise, and has been shown to prolong survival of these patients so much as to be comparable to surgery. PEI is so convenient and easy that it could be done at the patient's home even in poor countries where health systems are weak. Ethanol destroys cancer tissue and diffuses well in the cirrhotic liver, and the procedure can be repeated several times on the same nodule or on new ones. The side effects are minimal, no general anesthesia is required and the procedure can be performed in an ambulatory situation. The use of this simple and inexpensive procedure has shown that it is possible to prolong survival of many patients and allow them to maintain an excellent quality of life[106-112]. PEI can be used in patients with more than one nodule, usually no more than 3, and because it can easily be repeated on the already treated nodule, offers patients a simple and inexpensive treatment of their disease.

The ablative technique obtained by PEI has been improved by using RFA that can destroy cancer tissue in only one step. This procedure, more complex and expensive than PEI, has the advantage that it can treat cancer lesions with a larger diameter than PEI can (usually < 5 cm diameter) and many of them with one single application. Because of the pain involved in the procedure, RFA is more commonly performed with the patient under light anesthesia. Side effects occur more commonly than with PEI and but are rarely severe.

A comparison between PEI and RFA shows that when patients are carefully selected, the results in terms of overall survival are similar, though disease recurrence can be observed more frequently in patients treated by PEI[113,114].

Ablation of the tumor can be obtained by other means, such as acetic acid or laser, but what is crucial is the expertise of the operator and good patient selection.

PALLIATIVE TREATMENTS

As mentioned earlier, most HCC patients cannot undergo surgery and so, for them, especially those with locally advanced disease, the most effective and safe treatment is that which aims to destroy the most cancer tissue without affecting liver reserve function.

The procedures that can be used are PEI, RFA, TAE, TACE, in association or not with different forms of chemotherapy.

PEI and RFA are so easy and safe that they can be performed even in patients with advanced stage of liver cirrhosis (stage C according to the BCLC system). The main risks are infection and bleeding in patients with severe defects in blood coagulation (pro-thrombin time < 60% and platelet counts < 60000/mL^3). The risk of diffusing cancer cells along the needle passage is real,

but in our experience is a rare event.

In several patients where surgery is not indicated, TAE and TACE can be employed to embolize the artery feeding the HCC. Patients for this procedure must be selected very carefully and the individual performing the procedure needs to be expert (usually a radiologist) in superselective embolization[114,115]. Necrosis of the tumor is rarely complete because of tumor angiogenesis that forms bunches of new small vessels that feed the cancer nodule. In addition, in many cases significant blood supply comes from portal blood and the liver cancer cells are ischemia resistant[116,117]. Nevertheless, the growth of the tumor may be impaired for weeks, making TACE or TAE the most commonly performed pre-transplantation procedure and the most used technique in HCC of large dimension and/or with too many nodules to be ablated.

Several authors have reported TACE prolonging survival also in patients with intermediate or advanced HCC. Considering that there are no other possible procedures in many patients, chemoembolization must be regarded as palliative but also active treatment[118,119].

Recently, two retrospective works have compared liver resection and TACE in patients with BCLC stage B. In a Chinese study, resection appears as safe as TACE and provides better overall survival at 3 years (59% *vs* 29%) and 5 years (37% *vs* 14%) with a highly statistically significant difference ($P < 0.001$)[119]. In the other study, from Spain, the authors analyzed their results according to the revised BCLC classification system that divides stage B into 4 substages (B1, B2, B3 and B4) on the basis of Child Pugh score, inclusion in the Milan criteria and furthermore, size and number of lesions under or over 7, and Performance Status (P.S.) according to ECOG. They found that liver resection provides particularly good results in the B1 group (Child Pugh score 5-7, within Milan Criteria and < 7, and P.S. = 0) with an overall survival of 62.9% after 5 years and a recurrence rate of 25% as opposed to a recurrence rate of 60% in the TACE group ($P < 0.018$). B3 (Child Pugh score = 7, beyond Milan criteria or > 7 and P.S. = 0) or B4 (Child Pugh score 8 or 9, any Milan criteria or number, P.S. >0) patients had the worst prognosis after 5 years (15.4% as overall survival). These authors concluded that modern surgical techniques and good selection of patients with liver cirrhosis and HCC in BCLC stages B1 or B2 (Child Pugh 5 or 6, beyond Milan Criteria but number < 7, P.S. = 0) according to the new BCLC system can provide particularly good results with liver resection in terms of survival and recurrence rate compared to similar patients treated with TACE[120].

In the last decade, the use of radio-labeled microspheres or embolizing beads that carry anticancer agents such as doxorubicin or cisplatin has been proposed to treat advanced HCC. The results have been encouraging and similar to those obtained by traditional TACE or TAE in terms of anticancer activity. Side effects appear less frequent and of minor gravity, making this new BEAD-TACE procedure one of the most favored in many centers[121-124].

Since all these treatments must be considered palliative and locally active, to improve their efficacy, it has been proposed to associate them with systemic chemotherapy.

CHEMOTHERAPY

In intermediate or in selected advanced HCC patients who cannot be treated by ablative or embolizing techniques or where HCC is already extrahepatic, the only systemic chemotherapy that has been proved to be effective is sorafenib[125-127]. This drug (800 mg/d, orally) is a tyrosine kinase activity inhibitor that acts mainly as antiangiogenic to slow down the growth of the tumor. The response rate is not particularly high (about 4%) and toxicity may be relevant. Sorafenib has been described as decompensating liver cirrhosis, and then its dosage must be reduced to 600 mg/d or 400 mg/d. In a few cases therapy must be withdrawn and it is possible to reintroduce it at a lower dosage once liver function has recovered. Sorafenib can be too toxic in patients with advanced liver cirrhosis, provoking side effects that include mucositis, skin toxicity, fatigue, bleeding and liver failure[128,129]. It should be considered that drug resistance to sorafenib and other inhibitors of tyrosine-kinase activity has been reported[130]. It is also possible to try to reverse drug resistance by using other drugs that interfere with mechanisms of drug resistance[131]. Agents such as sunitinib, erlotinib, and many others have been proposed but toxicity or drug resistance have limited their use and they have been abandoned so far for the treatment of HCC.

Recently, as already mentioned when talking about the biology of HCC, and following experimental data showing the importance of the cMET pathway in HCC, a phase Ⅱ randomized study was carried out using tivantinib, a specific inhibitor of the cMET, as second-line treatment after sorafenib. The results of this study show that especially in HCC showing MET expression tivantinib is active and significantly slows tumor growth. The authors conclude that a phase Ⅲ study is warranted to see the real impact of this new drug in advanced HCC[55,56].

Several authors have tried to improve the efficacy of these palliative treatments by associating sorafenib with TACE, TAE, ablation, or surgery[132-135]. Sorafenib has also been used as adjuvant chemotherapy in patients after complete resection of HCC to prevent relapse or as neoadjuvant chemotherapy before liver transplantation or resection. Though some encouraging preliminary results have emerged, none of these studies have shown clear advantages and must be regarded as experimental protocols which need to be repeated as controlled and much larger studies[136].

Many years ago we performed a pilot study (phase Ⅱ) by using traditional intrahepatic artery chemotherapy using 5-fluorouracil in patients who were not suitable for surgery or ablative techniques or

after relapse of HCC. This drug was well tolerated and our results, in terms of tumor response and survival, were positive compared with historic controls and in line with what others have recently shown, but the lack of randomized controls and small number of patients enrolled strongly limited the overall significance of this study[137].

In conclusion, HCC is at present one of the most challenging cancers in clinical practice[138]. Though our understanding of its risk factors and how it develops have improved in the last decade, so much remains to be clarified. Currently the best way to significantly reduce the death rate remains prevention of HBV and HCV infection and alcoholism.

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