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World Journal of Hepatology
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8226 Regency Drive, Pleasanton, CA 94588, USA
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MINIREVIEWS

Nutritional evaluation in cirrhosis: Emphasis on the phase angle

Sabrina Alves Fernandes, Angelo Alves de Mattos, Cristiane Valle Tovo, Claudio Augusto Marroni

Sabrina Alves Fernandes, Angelo Alves de Mattos, Cristiane Valle Tovo, Claudio Augusto Marroni, Postgraduate Program at Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, RS 90420-060, Brazil

Sabrina Alves Fernandes, Post Graduation Program in Bioscience and Rehabilitation and the Post Graduation Program in Rehabilitation and Inclusion, Methodist University - IPA, Porto Alegre, RS 90420-060, Brazil

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Correspondence to: Dr. Sabrina Alves Fernandes, PhD, Postgraduate Program at Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Rua Professor Duplan 72/01, Porto Alegre, RS 90420-060, Brazil. sabrinaafernandes@gmail.com
Telephone: +55-51-33038795
Fax: +55-51-33038795

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Abstract

Protein-calorie malnutrition (PCM) is a common condition in cirrhotic patients, leading to a worse prognosis, complications, poor quality of life and lower survival rates. Among ways of assessing nutritional status, there are anthropometric methods such as the evaluation of the triceps skinfold, the arm circumference, the arm muscle circumference and the body mass index, and non-anthropometric methods such as the subjective global assessment, the handgrip strength of non-dominant hand, and the bioelectrical impedance analysis (BIA). PCM is frequently under-diagnosed in clinical settings in patients with cirrhosis due to the limitations of nutritional evaluation methods in this population. BIA is a useful method, but cannot be indicated in patients with abnormal body composition. In these situations, the phase angle (PA) has been used, and can become an important tool in assessing nutritional status in any situation. The PA is superior to anthropometric methods and might be considered as a nutritional indicator in cirrhosis. The early characterization of the nutritional status in patients with cirrhosis means an early nutritional intervention, with a positive impact on patients' overall prognosis. Among the usually accepted methods for nutritional diagnosis, the PA provides information in a quick and objective manner.

Key words: Malnutrition; Bioelectrical impedance; Phase angle; Sarcopenia; Nutrition

Core tip: Malnutrition in cirrhotic patients is a common clinical condition, but there is currently no nutritional diagnosis method defined as the gold standard. Presently, the only nutritional indicator compatible with the clinical condition through the Child-Pugh score in cirrhosis is the phase angle (PA). The PA has been

a reliable method and is free of influences regarding changes in body composition of cirrhotic patients at an advanced stage. The PA measured by bioelectrical impedance analysis promises to be a significant parameter for early nutritional intervention in patients with chronic liver disease.

Fernandes SA, de Mattos AA, Tovo CV, Marroni CA. Nutritional evaluation in cirrhosis: Emphasis on the phase angle. *World J Hepatol* 2016; 8(29): 1205-1211 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i29/1205.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i29.1205

INTRODUCTION

Many factors favor the development of protein-calorie malnutrition (PCM) in cirrhosis, a common condition that leads to serious repercussions regarding the general state and clinical course of patients[1] and that presents a worse prognosis, complications, poor quality of life and lower survival rates[2,3]. However, malnutrition is often underdiagnosed in this situation[4]. It is difficult to evaluate the nutritional status of patients with cirrhosis, as there are particularities due to the clinical condition that make it hard to precisely inform the real nutritional status and its consequent prognosis[5].

Malnutrition can be directly related to a poor survival rate in patients with cirrhosis, and its improvement is a strong indicator of quality of life, especially for those who are on the waiting list for liver transplantation[6]. Early detection of malnutrition in cirrhotic patients is of great clinical relevance and interferes positively in patient recovery[7,8].

The new European Society of Clinical Nutrition and Metabolism (ESPEN) consensus[9], recommends that subjects at risk of malnutrition be identified by validated screening tools, and they advocate two options for the diagnosis of malnutrition: The body mass index (BMI, kg/m^2) lower than 18.5 to characterize malnutrition, and the combined finding of unintentional weight loss and either reduced BMI or a low fat-free mass index (FFMI), or both. Weight loss could be either greater than 10% of habitual weight regardless of time, or greater than 5% over 3 mo. Low FFMI is characterized as lower than 15 or lower than 17 kg/m^2 in females or males, respectively. However, many other tools, such as anthropometric and non-anthropometric methods as well as laboratory tests may be used, classifying the degrees of malnutrition as mild, moderate and severe in different ways - although none of these other tools are widely recognized as a gold standard, and thus must be considered together. Various nutritional parameters has been used to assess the nutritional status such as anthropometry parameters [mid arm circumference, triceps skinfold thickness (TSF)], hand grip, serum albumin level, creatinine height index, and total lymphocyte count[8-10]. Recently electrical bioimpedance has been proposed for body composition analysis of patients with chronic liver disease[8,11-13]. In view of paucity of data on prevalence of malnutrition and its relationship with morbidity and mortality in patients with liver cirrhosis as well as the absence of a gold standard method for nutritional evaluation in these patients, we conducted this study to determine the prevalence of malnutrition by various methods and its clinical importance in cirrhotic patients according the severity of disease.

Considering the scarcity of data in the evaluation of malnutrition in patients with liver cirrhosis as well as the absence of a gold standard for nutritional evaluation in these patients, the present review was performed, critically addressing the following points: Malnutrition in cirrhosis; sarcopenia; nutritional assessment in cirrhosis; bioelectrical impedance analysis and the phase angle[10].

MALNUTRITION IN CIRRHOSIS

Malnutrition is one of the most frequent complications in cirrhotic patients[11]. However, its frequency in cirrhosis is highly variable, and may affect between 20% of the patients with compensated cirrhosis and more than 60% of these patients with severe hepatic disfunction[12,13].

Alberino *et al*[6] studied 212 hospitalized patients with liver cirrhosis that were followed for 2 years or until death. The severely and moderately malnourished patients had lower survival rates than normal and over nourished patients, and severe depletion of muscle mass and body fat was found to be an independent predictor of survival. This data suggests that malnutrition is an independent predictor of survival in patients with liver cirrhosis. Additionally, a nationwide analysis of the prevalence of PCM in patients with cirrhosis and portal hypertension (PHTN) and its mortality was conducted in the United States[13]. There were 114703 admissions with cirrhosis and PHTN between 1998 and 2005, and the prevalence of PCM was higher among patients with cirrhosis and PHTN compared with general medical inpatients; this prevalence was also associated with higher in-hospital mortality and resource utilization. The authors concluded that PCM may be an indicator of disease severity and should be routinely assessed on admission.

Besides the metabolic changes observed in cirrhosis, there are factors that can contribute to increased malnutrition in this population. Factors such as anorexia and early satiety, triggered by changes in endogenous leptin, mineral deficiencies and reduction in gastric expandability favor a negative energetic balance, with an imbalance between ingestion and energy intake and expenditure, and PCM may develop as a result[14]. The zinc and magnesium deficiencies that may be often seen in the population of patients with cirrhosis contribute to the development of dysgeusia, which aggravates the intake capacity[15,16].

The clinical complications that can occur in decompensated cirrhosis - such as gastrointestinal bleeding,

hepatic encephalopathy (HE) and ascites - can further accentuate the PCM situation[14], alongside the diet offered to these patients, which is restrictive in most cases. Thus, although for a short period of time, a hypoproteic diet may be eventually implemented, especially in cases of HE grades Ⅲ and Ⅳ[4]. It is worth highlighting that the protein restriction has been banned in HE in order to prevent the worsening of PCM, and a diet with 1 to 1.5 g of protein per kilogram of weight is suggested[4].

The recommended low-sodium diet in the treatment of patients with ascites and peripheral edema makes the food intake even more difficult and significantly decreases the daily calorie intake, thus stimulating PCM. A low-sodium diet with a daily intake of 2 g of salt is recommended[17].

It is known that skeletal muscles contribute in the proper metabolic functioning of macro and micronutrients, favoring body homeostasis. In individuals with cirrhosis, there is a significant loss and dysfunction of such musculature, often characterizing sarcopenia[18,19]. This state generates systemic and inflammatory changes associated with the PCM, negatively impacting the patient's clinical status[20].

The classical study by Merli *et al*[21] prospectively evaluated a total of 1053 cirrhotic patients to determine whether malnutrition is a risk factor for mortality in cirrhosis. They found that the cumulative survival was lower in patients with a reduction in muscle mass in Child-Pugh classes A and B.

Montano-Loza *et al*[22] studied 112 cirrhotic patients consecutively evaluated for liver transplantation, and observed that sarcopenia occurred in up to 40% of the patients and was related to the worsening of clinical conditions represented by biochemical and clinical parameters; moreover, by multivariate Cox analysis, the Child-Pugh (HR = 1.85; P = 0.04), the model for end-stage liver disease (MELD) scores (HR = 1.08; P = 0.001) and sarcopenia (HR = 2.21; P = 0.008) were independently associated with mortality. The median survival time for patients with sarcopenia was 19 ± 6 mo, compared with 34 ± 11 mo among non-sarcopenic patients (P = 0.005). Sarcopenia can be considered an indicator of risk of infection in cirrhotic individuals, directly reflecting a decline in immune function, worsening the quality of life and decreasing survival.

SARCOPENIA

Malnutrition in cirrhosis is closely related to the development of sarcopenia, which will be one of the most common complications related to survival in this population of patients. Nevertheless, there is a lack of an optimal index for sarcopenia and of a consensus definition for sarcopenia in patients with cirrhosis in whom ascites and edema may interfere with body composition analysis[23].

Sarcopenia is a syndrome characterized by progressive and generalized loss of skeletal muscle mass and strength with a risk of adverse outcomes such as physical disability, poor quality of life and death[24,25]. The European Working Group on Sarcopenia in Older People recommends using the presence of both low muscle mass and low muscle function (strength or performance) for the diagnosis of sarcopenia[26].

Montano-Loza *et al*[19] evaluated a population of 248 cirrhotic patients enlisted for liver transplantation and identified sarcopenia in 45% of patients; sarcopenia was associated with a longer period of hospitalization and higher risk of bacterial infection after transplantation.

Similarly, Tandon *et al*[5] evaluated 142 patients with cirrhosis listed for liver transplantation, and found that 41% were sarcopenic. Male gender, the BMI, and Child-Pugh class C cirrhosis (but not the MELD score) were independent predictors of sarcopenia, which was an independent predictor of mortality after adjustments for age and MELD scores. The authors concluded that sarcopenia is associated with increased waiting-list mortality and is poorly predicted by subjective nutritional assessment tools such as BMI and subjective global assessment (SGA). The objective assessment of sarcopenia holds promise for prognostication in this patient population.

NUTRITIONAL ASSESSMENT IN CIRRHOSIS

Among the different ways of assessing nutritional status, there are anthropometric methods such as determining the TSF, the arm circumference (AC), the arm muscle circumference (AMC) and the BMI, as well as non-anthropometric methods such as SGA, handgrip strength (HS) of non-dominant hand, the adductor pollicis muscle thickness (APMT) and the phase angle (PA) by bioelectrical impedance analysis (BIA).

Classical anthropometry assesses the measurement of body size and its proportions. The results obtained are compared with the points of reference previously described[27].

Cirrhotic individuals present significant changes regarding body weight by hydric retention, making BMI an inadequate method for nutritional diagnosis[28]. Such distortion was observed in the study performed by Gottschall *et al*[29], in which 61.8% of patients were classified as overweight, while other techniques, such as SGA or HS, found malnutrition in 38% and 85.7% in the same population of patients, respectively.

The TSF measurement indirectly estimates fat mass by measuring the thickness of two layers of skin and the adjacent subcutaneous fat. This is a good assessment method, although some studies have found a low prevalence of malnutrition in cirrhosis when comparing this method to others[30-32].

Abbott *et al*[33] and Alberino *et al*[6] described, in their studies, that 54% of the evaluated cirrhotic patients were malnourished when utilizing AC and AMC, supporting the findings of Merli *et al*[21], which suggest

AMC as an accurate indicator of malnutrition in patients in the early stages of cirrhosis. On the other hand, a study performed in our center by Fernandes *et al*[34] showed that AC and AMC are not sensible parameters for the nutritional diagnosis.

As a general rule, the anthropometric parameters may be affected when there is hydric retention; the results are observer-dependent and can be conflicting, becoming inadequate in the nutritional assessment of patients with cirrhosis.

When considering the non-anthropometric methods, SGA is a method of interest that uses easily reproducible parameters such as the clinical history and physical conditions of the individual, focusing on the nutritional aspects and offering a score that provides the nutritional diagnosis[35]. However, this method shows limitations, especially when the patient has some difficulty to understanding or even HE, as patients will not report their nutritional history adequately[36].

Figueiredo *et al*[37] observed that SGA has a sensitivity of only 22% in cirrhotic individuals and underestimates their nutritional status in 57%, while overestimating it in 6%[38]. On the other side, Ritter and Gazzola[38] established SGA as a good option for the nutritional assessment of patients with liver disease.

Although some authors[36,39,40] have suggested that SGA might be useful to assess the nutritional status evolution of cirrhotic patients who are liver transplant candidates, these studies have detected malnutrition in only 25% of cases with this method.

The HS assessment through dynamometry refers to the measurement of muscle strength and of pressure distribution[41], classifying the nutritional status of individuals by gender and age. In dynamometry, there is the assumption that in PCM there is a decrease in muscle mass, hindering one's functional capacity[42]. Studies with cirrhotic patients have shown the superiority of HS assessment when compared to SGA in diagnosing malnutrition; HS is considered a low-cost and simple method that is not influenced by the presence of hydric retention[42]. Curiously, in different studies, HS - while proving to be a good method in assessing nutritional risk - does not present a correlation between malnutrition and the staging of liver disease through the Child-Pugh score, although it is considered that liver disease patients, when classified as Child-Pugh C, are malnourished *per se*[41].

The APMT has been suggested as a promising marker of muscle mass[43,44]. The adductor pollicis muscle is the only muscle that allows direct thickness assessment, as it is anatomically well defined and flat in shape[45]. However, few have looked into it as a marker of nutritional status[46].

BIOELECTRICAL IMPEDANCE ANALYSIS

The BIA is a method for assessing body composition that has shown good results regarding the nutritional state, as it shows fat mass, lean mass and basal metabolic rate, in addition to total body water in healthy subjects[47]. The distribution of body fat has an important influence in the severity of certain diseases, such as in cardiovascular disease and depending on the type of fat mass distribution, may pose a higher risk of developing tumors[48]. Thus, in addition to providing a nutritional status assessment, BIA can also be a good prognosis method that is characterized as a practical, quick, non-invasive and low-cost method[48,49].

In the clinical nutritional assessment of a cirrhotic patient, it is possible to perform compartmentalized body assessment through BIA not only in the classical model that is normally used (fat mass and fat-free mass), but also in a quantitative manner, obtaining cellular distribution and providing information on body composition[50].

In the past, there were restrictions on the use of BIA for individuals with abnormal body composition; that is, amputations, electrolyte disorders (edema and ascites), obesity, dystrophies and pregnancy, because the BIA assumes that the human body resembles a cylinder of constant hydration and invariably lean mass[47,51].

Some tissues with high water and electrolyte composition - such as cerebrospinal fluid, blood or muscles - are high electrical conductors. On the other hand, fatty tissues or bones are highly resistant to electric current[47]. The conductivity of biological tissues is virtually ionic, meaning that electric charges are transferred by the ionization of the salts, bases or acids in body fluid. Thus, organic conductivity is directly proportional to the quantity of body fluid volume. Therefore, if the patient is in a state of overhydration, the amount of lean body mass is overestimated, modifying the result of the body assessment, which is one of the limitations of this method[47].

For the assessment of nutritional state by BIA, there are monofrequencial or multifrequencial portable equipment, differing on the options of the amperage of the electric current to allow greater sensitivity of the examination. The patient remains in dorsal decubitus position, with hands and legs parallel to the body. One electrode is placed on the dorsal hand, at the middle finger level, and one in the wrist joint, both on the right side. Another pair of electrodes is placed on the dorsal foot, at the middle toe level, and in the ankle joint, also on the right side. The electrical current enables measuring resistance and reactance and obtaining the PA value.

THE PHASE ANGLE

In view of the limitations of BIA, the clinically established bioelectrical impedance parameter is the PA. The PA was originally described by Baumgartner *et al*[51] for the diagnosis of metabolic disorders. The data is obtained through BIA and is directly calculated through the arc tangent formula (Xc/R). The tissues' capacitance (Xc) is related to cellularity, cell-size and integrity of cellular membrane. The resistance (R) is dependent on the hydration state of the tissues. The ratio of components results in a geometric graphic, where the ratio of R and

Xc results in an angle called the PA.

BIA is represented by the vector Z, which is a combination of the perpendicular vectors R and Xc. The vector Z has a module M, and the horizontal axis defines the PA[52].

The PA reflects the cellular vitality and integrity, where normal values (according to gender and age) indicate preserved cellular activity[34,53,54], being highly predictive of clinical progression in a number of diseases[55].

It has been suggested that the PA can become an important tool in assessing nutritional status in any situation, being superior to anthropometric and biochemical methods[44].

There are reference values according to age and gender[8], and some authors prefer to establish cutoff points according to the disease being studied[47].

The PA has also been studied as a prognostic marker in different clinical situations, such as tumors, acquired immunodeficiency syndrome, and heart and liver diseases[54].

In a review, Llames *et al*[55] concluded that the PA may be sufficient to monitor the nutritional status of an individual. In a population-based study, they observed a higher PA in men than in women, except in individuals over 70 years of age. When stratified by age and gender, the values tend to increase as BMI increases in values of up to 35 kg/m^2; however, there is a decrease in PA in groups with BMI above 35 kg/m^2[55].

There are few studies evaluating the PA in cirrhotic patients.

Selberg *et al*[56], in a prospective study of 305 patients with cirrhosis, correlated the PA with muscle mass, muscle strength, and survival rates. They observed that patients with a PA equal to or lower than 5.4 degrees showed lower survival rates than those with PA values above 6.6 degrees. In those with PA under 4.4 degrees, survival was even (and significantly) lower. Variables such as total body potassium, anthropometric measurements and BIA were evaluated separately; however, only the PA proved to be an isolated predictor of survival. The authors concluded that the PA appears to be superior to conventional methods in the clinical assessment of patients with cirrhosis.

In a retrospective study, Pirlich *et al*[53] evaluated the cellular mass composition of 41 cirrhotic patients (20 with ascites and 21 without) through BIA, which was considered the reference method. The study shows that the PA is a tool that is able to detect body cellular mass and to identify its decrease in cirrhotic patients. The PA offers reliable PCM estimates even in patients with large amount of ascites, proving to be superior to commonly used techniques.

In a cohort that assessed 66 cirrhotic patients stratified by their clinical condition through the Child-Pugh score and followed-up during a 17-mo period, the established PA for this population was 5.18 degrees. Patients with values below this angle were considered to have poor prognosis and shorter survival rates. It is worth highlighting that as the patients' clinical situation worsened, the PA decreased, showing a prognostic value[57].

Corroborating these findings, we assessed the nutritional status of 129 cirrhotic patients through different methods and demonstrated that the only method that is able to correlate malnutrition with the staging of liver disease, evaluated through the Child-Pugh classification, was the PA. We set the PA cutoff point as 5.4 degrees, and patients with values below this discriminatory level showed a worse prognosis. We should point out the discrepancies between the results of different evaluation methods (anthropometry, HGS and BIA) used to diagnose PCM, once the diagnosis for malnutrition may vary from 5.4% to 69.3% in the same population, depending on the assessment method employed[34]. The PA evaluated through the BIA presented a sensitivity and specificity of 68.9%-70.0% and 49.2%-56%, respectively, when compared to the HGS[34].

Later, another study performed in our center evaluated 195 cirrhotic patients, reinforcing the idea that the PA is a good prognostic marker when compared to other methods, as it is the only one that correlates with the real clinical condition of the patient[58].

Recently, Ruiz-Margáin *et al*[59] assessed 249 compensated cirrhotic patients in a prospective cohort study with a 48-mo follow-up period. The PA cutoff point for malnutrition was lower than or equal to 4.9 degrees. This study also concluded that the PA is a good prognostic marker, associating the PCM with mortality rate.

A cohort study conducted in our center evaluated 32 cirrhotic patients enlisted for liver transplantion[36]. The patients were interviewed and evaluated on the day of or on the day before the transplant, and 1, 6, and 12 mo after surgery. The assessment of nutritional status was performed applying diagnostic procedures in sequence: Anthropometry, HS, APMT and PA. Methods that better demonstrated the real prevalence of malnourished patients before transplantation were PA (25%), AMC (21.9%) and AC (18.8%). The percentage of malnourished patients was significantly higher after 1 mo of transplantation when compared to the percentage in 6 mo and 1 year after transplantation. It was suggested that the PA could be widely used with this population, since the results are consistent, reliable and reproducible.

Wagner *et al*[60] evaluated nutritional methods that informed the nutritional status of 71 post-transplantation patients. Patients were divided into 3 groups according to time since transplantation: 5 years, between 5 and 10 years, and over 10 years. They used the PA cutoff point as below 5 degrees in order to diagnose malnutrition. The PCM diagnosis was made in 81.2%, 31.6% and 31.7% in each group, respectively (P = 0.008). In this study, the PA showed a higher prevalence of malnutrition among the population of patients in the first years after liver transplantation.

CONCLUSION

The cirrhotic patient is malnourished *per se*, regardless of etiology and the severity of the disease. The early characterization of the nutritional status in patients with cirrhosis means an early nutritional intervention, with a positive impact on patients' overall prognosis. Compared to the usually accepted methods for nutritional diagnosis, the PA obtained through BIA is the only appropriate method to evaluate the nutritional status of cirrhotic, providing safe information in a quick and objective manner as a prognostic index.

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ORIGINAL ARTICLE

Basic Study

Potential role of killer immunoglobulin receptor genes among individuals vaccinated against hepatitis B virus in Lebanon

Nada M Melhem, Rami A Mahfouz, Khalil Kreidieh, Rabab Abdul-Khalik, Rolla El-Khatib, Reem Talhouk, Umayya Musharrafieh, Ghassan Hamadeh

Nada M Melhem, Khalil Kreidieh, Rolla El-Khatib, Medical Laboratory Sciences Program, Faculty of Health Sciences, American University of Beirut, Beirut 1107-2020, Lebanon

Rami A Mahfouz, Rabab Abdul-Khalik, Department of Pathology and Laboratory Medicine, Faculty of Medicine, American University of Beirut, Beirut 1107-2020, Lebanon

Reem Talhouk, Department of Health Management and Policy, Faculty of Health Sciences, American University of Beirut, Beirut 1107-2020, Lebanon

Umayya Musharrafieh, Ghassan Hamadeh, Department of Family Medicine, Faculty of Medicine, American University of Beirut, Beirut 1107-2020, Lebanon

Author contributions: Melhem NM designed the study, performed the analysis and wrote the paper; Mahfouz RA contributed to the analysis; Kreidieh K and Abdul-Khalik R performed the experiments; El-Khatib R, Talhouk R, Musharrafieh U and Hamadeh G helped in the recruitment of participants.

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Correspondence to: Dr. Nada M Melhem, PhD, Associate Professor of Infectious Diseases and Microbiology, Medical Laboratory Sciences Program, Faculty of Health Sciences, American University of Beirut, 11-0236 Riad El Solh, Beirut 1107-2020, Lebanon. melhemn@aub.edu.lb
Telephone: +961-1-350000-4699
Fax: +961-1-744470

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Abstract

AIM

To explore the role of killer immunoglobulin receptor (*KIR*) genes in responsiveness or non-responsiveness to vaccination against hepatitis B virus.

METHODS

We recruited 101 voluntary participants between March 2010 and December 2011. Sera samples from vaccinated and non-vaccinated participants were tested for the presence of anti-HBs antibodies as a measure of protection against hepatitis B, hepatitis B surface antigen and hepatitis B core antibody as indicators of

infection by enzyme-linked immunosorbent assay. *KIR* gene frequencies were determined by polymerase chain reaction.

RESULTS

Sera samples from 99 participants were tested for the levels of anti-HBs as an indicator of protection (≥ 10 mIU/mL) following vaccination as defined by the World Health Organization international reference standard. Among the vaccinated participants, 47% (35/74) had anti-HBs titers above 100 mIU/mL, 22% (16/74) had anti-HBs ranging between 10-100 mIU/mL, and 20% (15/74) had values of less than 10 mIU/mL. We report the lack of significant association between the number of vaccine dosages and the titer of antibodies among our vaccinated participants. The inhibitory KIR2DL1, KIR2DL4, KIR3DL1, KIR3DL2, and KIR3DL were detected in more than 95%, whereas KIR2DL2, KIR2DL3, KIR2DL5 (KR2DL5A and KIR2DL5B) were expressed in 56%, 84% and 42% (25% and 29%) of participants, respectively. The observed frequency of the activating *KIR* genes ranged between 35% and 55% except for KIR2DS4, detected in 95% of the study participants (40.6% 2DS4*001/002; 82.2% 2DS4*003/007). KIR2DP1 pseudogene was detected in 99% of our participants, whereas KIR3DP*001/02/04 and KIR3DP1*003 had frequencies of 17% and 100%, respectively. No association between the frequency of *KIR* genes and anti-HBs antibodies was detected. When we compared the frequency of *KIR* genes between vaccinated individuals with protective antibodies titers and those who lost their protective antibody levels, we did not detect a significant difference. KIR2DL5B was significantly different among different groups of vaccinated participants (group Ⅰ > 100 mIU/mL, group Ⅱ 10-100 mIU/mL, group Ⅲ < 10 mIU/mL and group Ⅳ with undetectable levels of protective antibodies).

CONCLUSION

To our knowledge, this is the first study screening for the possible role of *KIR* genes among individuals vaccinated against hepatitis B virus (HBV). Our results can be used to design larger studies to better understand the role of *KIR* genes in protection against or susceptibility to HBV post vaccination.

Key words: Hepatitis B virus; Killer immunoglobulin receptors; Hepatitis B vaccine; Lebanon; Natural killer cells

Core tip: Currently, there are no data supporting the use of booster doses of hepatitis B vaccine among immuno-competent individuals responding to a complete primary vaccination regimen. Importantly, 5%-10% of healthy adults do not generate protective levels of antibodies and are hence considered non-responders. This study aims to explore the role of killer immunoglobulin receptor genes in responsiveness or non-responsiveness to vaccination against hepatitis B virus.

Melhem NM, Mahfouz RA, Kreidieh K, Abdul-Khalik R, El-Khatib R, Talhouk R, Musharrafieh U, Hamadeh G. Potential role of killer immunoglobulin receptor genes among individuals vaccinated against hepatitis B virus in Lebanon. *World J Hepatol* 2016; 8(29): 1212-1221 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i29/1212.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i29.1212

INTRODUCTION

Infection with hepatitis B virus (HBV) results in a spectrum of clinical outcomes ranging from acute hepatitis to end-stage liver disease and hepatocellular carcinoma[1] with an estimated lifetime risk of 25%-40%[2]. Booster studies suggest that memory begins to decline 15 years following vaccination among adolescents vaccinated in infancy[3-6]. Other studies suggest the persistence of immune memory for 20 years or longer[7-9]. Currently, there are no data supporting the use of booster doses of hepatitis B vaccine among immuno-competent individuals responding to a complete primary vaccination regimen (3 doses). Importantly, 5%-10% of healthy adults do not generate protective levels of antibodies and are hence considered non-responders[10]. Consequently, long-term protection is still debatable[11] and not linked to genetic factors.

Natural killer (NK) cells are known to induce antiviral and antitumor immunity *via* production of pro-inflammatory cytokines and lysis of infected or transformed cells[12]. Killer immunoglobulin receptor (*KIR*) genes encode receptors expressed on NK cells. Based on the gene content, two groups of *KIR* haplotypes are known in humans: A and B. Haplotype A encodes inhibitory receptors and consists of nine genes (3DL3, 2DL3, 2DP1, 2DL1, 3DP1, 2DL4, 3DL1, one activating (2DS4), 3DL2, and 2DL5) whereas haplotype B carries a variety of gene combinations and encodes more activating receptors as compared to haplotype A (3DL3, 2DS2, 2DL2, 2DL5B (inhibitory) 2DS3, 2DP1, 2DL1, 3DP1, 2DL4, 3DS1, 2DL5A (inhibitory), 2DS5, 2DS1, and 3DL2)[13].

KIR3DS1- and KIR3DL1-expressing NK cells were reported to expand in acute and chronic human immunodeficiency virus (HIV)-1 infection, respectively[14]. Similarly, reports suggest that KIR2DL2 and/or KIR2DL3 along with their ligand human leukocyte antigen (HLA)-C1 are associated with severe influenza infection; in addition, the frequency of KIR3DS1, KIR2DS5 and KIR2DL5 was also related to the severity of the disease[15]. KIR2DS2 and KIR2DS3 were found to be associated with susceptibility to chronic hepatitis B infection, whereas KIR2DS1, KIR3DS1 and KIR2DL5 may act as protective genes leading to viral clearance among the Chinese Han population[16]. A difference between the frequency of

different *KIR* haplotypes among chronically infected individuals and those spontaneously recovering from HBV infection was also demonstrated in this population[17]. Recently, the rates of KIR2DL3 and 3DS1 were reported to be higher in healthy Turkish individuals as compared to patients with chronic HBV and those with spontaneous remission[18]; authors suggested the possible role of these genes in protection against HBV infection. In addition, genetic factors have been reported to play a role in the regulation of post-vaccine immune responses[19]. This was observed with antibody responses to a number of vaccine antigens including hepatitis B.

It is clear that the interaction between KIRs and their corresponding HLA ligands is implicated in differential responses to HIV, hepatitis C virus (HCV) and HBV as well as other disease conditions[20-23]. Immune responses, like many biological responses, are characterized by a wide range of variation between individuals. This has been described following natural infection or in response to vaccination[24]. HLA, cytokines, toll-like receptors and related gene variants have been associated with a variety of immune responses following vaccinations. Recently, single-nucleotide polymorphism associations were described to be involved in innate and adaptive immune response regulation following measles and rubella vaccinations[25,26]. Similarly, a difference in gene expression was also reported between high and low responders to smallpox vaccine[27]. The fact that antibody response to hepatitis B vaccine is non-protective in up to 10% of individuals[10], and that a genetic basis to non-responsiveness is reported[19,24], prompted us to explore the role of *KIR* genes in response to hepatitis B vaccine in a cohort of healthy vaccinated Lebanese adults.

MATERIALS AND METHODS

Study participants and samples

Human subject approval was obtained for this study from the institutional review board of the American University of Beirut and all the methods were carried out in accordance with the approved ethical guidelines. A written informed consent was signed by the study subjects before participation in the study. A data collection form was administered to the study participants (≥ 18 years old) to collect demographic information, data related to exposure and risk behavior information. One hundred and one subjects were recruited during the time period March 2010-December 2011. Subjects were excluded if they had a prior or current history of HCV, HIV-1, renal disease or cancer. Children or adolescents of HBV carrier mothers and vaccinated in infancy were not included in the study. Blood was drawn from the study participants and peripheral blood mononuclear cells[28] and sera were collected and stored in liquid nitrogen and at -80 ℃, respectively. DNA was extracted from whole blood of the study participants using the QIAamp DNA Blood Midikit (Qiagen, Germany), as per manufacturer's instructions. The integrity of the purified DNA was checked by gel electrophoresis and storage was at -20 ℃.

Enzyme-linked immunosorbent assay

While we recruited 101 voluntary participants, sera samples of 99 HBV vaccinated and non-vaccinated study participants were tested in duplicate for the presence of anti-HBs antibodies as a measure of protection against hepatitis B, hepatitis B surface antigen (HBsAg) and hepatitis B core antibody (anti-HBc) as indicators of infection. We did not have enough sera to use in the analysis for two of our study participants. The Monolisa HBsAg ULTRA, anti-HBs PLUS and anti-HBc PLUS assays (BIO-RAD, France) were used as per manufacturer's instructions, respectively. Anti-HBs antibodies were measured in mIU/mL and levels ≥ 10 mIU/mL were indicative of post-vaccination protection[29,30].

KIR genotyping

The polymerase chain reaction (PCR)-based *KIR* genotyping SSP Kit (Invitrogen, Brown Deer, WI, United States) was used to detect the presence and absence of *KIR* genes, as per manufacturer's instructions. Briefly, 25 μL of DNA was used along with the primer sets to amplify the alleles described by the World Health Organization (WHO) international nomenclature committee (http://www.ebi.ac.uk/ipd/kir/). All amplifications were performed using PX2 thermocycler (ThermoHybrid, United Kingdom) programmed with a 1-min denaturation step at 95 ℃, followed by 30 cycles of 94 ℃ for 20 s, 63 ℃ for 20 s, and 72 ℃ for 90 s and finally 4 ℃ in the thermal cycler. PCR products were gel-purified and visualized under UV transillumination (Sigma, California, United States)[31]. The presence and absence of the following gene loci and variants were tested: 2DL1, 2DL2, 2DL3, 2DL4, 2DL5A, 2DL5B, 2DS1, 2DS2, 2DS3, 2DS5, 3DL1, 3DL2, 3DL3, 3DS1, 2DP1, and 3DP1. The variants of the KIR3DP1 pseudogene, KIR3DP*001/002/004 and KIR3DP1*003 were also detected in addition to KIR2DS4 variants: 2DS4*001/002 and 2DS4*003/007. The frequency of *KIR* was calculated by direct count of the observed phenotype and referred to as observed frequency (OF). In addition, the estimated *KIR* gene frequency (KF) for the putative loci was calculated using the following formula: KF = 1 - $\sqrt{}$ (1-OF) based on the assumption of Hardy-Weinberg equilibrium[32]. The frequencies of haplotype *A* and *B* were calculated using the following formula: haplotype $A = (2n_{AA} + n_{AB})/2n$ and haplotype $B = (2n_{BB} + n_{AB})/2n$, where n_{AA}, n_{AB} and n_{BB} are the numbers of individuals with haplotype group AA, AB and BB, respectively and n is the total number of individuals[33].

Statistical analysis

SPSS 19 was used for statistical analyses. We compared vaccinated and non-vaccinated subjects for each of the *KIR* polymorphisms using χ^2 and Fisher-exact test (FET) and reported the odds ratio and 95%CI. Similar analyses

Table 1 Demographics and characteristics of participants

	n	%
Gender (*n* = 101)		
Male	39	38.6
Female	62	61.4
Age (yr) (*n* = 95)		
19-29	38	40.0
30-39	19	20.0
40-49	17	17.9
50-59	11	11.6
60-69	6	6.3
70-79	2	2.1
80-89	2	2.1
Education (*n* = 99)		
Illiterate	3	3.0
Primary School Education	3	3.0
Secondary School Graduate	9	9.1
High School Education	10	10.1
University Undergraduate Level	50	50.5
Others	24	24.2
Occupation (*n* = 99)		
Student	15	15.2
Employed	74	74.7
Unemployed	7	7.1
Retired	3	3

were conducted for the comparison of protected and non-protected subjects within the vaccinated group. We also examined the relationship between genotypes and the presence or absence of *KIR* genes and the levels of anti-HBs and *KIR* genes among the vaccinated subjects using χ^2 and FET; for these comparisons, post-hoc tests were conducted only if the omnibus test was significant. We corrected for multiple comparisons for post-hoc tests using Bonferroni correction.

RESULTS

Characteristics of study participants

One hundred and one subjects were recruited between March 2010 and December 2011; 39% of the study participants were males and 61% were females. The majority of our study participants were 19-29 years old (40%). Table 1 summarizes the characteristics of the study participants. The majority (75%) held a university undergraduate degree or higher and was employed. During recruitment and when participants were asked about their vaccine status, 50% of our voluntary participants self-reported that they were vaccinated against HBV whereas 25% thought they were not vaccinated and 26% did not know their vaccine status.

In an attempt to confirm the vaccination status of our study participants, sera samples from 99 participants were tested for the levels of anti-HBs as an indicator of protection ($\geqslant$ 10 mIU/mL) as defined by the WHO international reference standard[30,34]. This is especially due to the lack of documented dosages of hepatitis B vaccine for many of the study participants as well as lack of knowledge of the vaccination status of many of the study participants. In the subsequent analyses, data on these 99 voluntary subjects are reported; participants with anti-HBs antibodies $\geqslant$ 10 mIU/mL will be considered vaccinated against hepatitis B. We also tested the sera samples for anti-HBc as a marker of previous infection and for HBsAg, a marker associated with recent exposure to HBV. All our participants were negative for HBsAg. Among the voluntary participants, 74/99 (75%) were vaccinated against hepatitis B as judged by the detection of anti-HBs titers; whereas 25% (25/99) were classified as non-vaccinated against hepatitis B. Among the vaccinated participants, 47% (35/74) had anti-HBs titers above 100 mIU/mL, 22% (16/74) had anti-HBs ranging between 10-100 mIU/mL, and 20% (15/74) had values of less than 10 mIU/mL. The time of vaccination (when available) ranged between the years 1999 and 2011, with some participants receiving 2 doses and others receiving 3 or more doses. When we tested for an association between the age groups of our study participants (19-29, 30-39, 40-49, 50-59, 60-69, 70-79 and 80-89) and the concentration of anti-HBs antibodies among vaccinated subjects, a *P* value of 0.047 was detected (FET). Nine percent (7/74) of the vaccinated participants had undetectable levels of protective antibodies. Five out of 7 (71%) of the former group were health-care workers; the latter are expected to be continuously monitored for protection against HBV due to the nature of their work. Importantly, anti-HBc was positive in 3% (3/99) of our study participants presenting with anti-HBs levels ranging between 110-1000 mIU/mL. This is associated with protection as a result of natural infection. One of these participants is a vaccinated female nurse, whereas the other 2 are non-vaccinated and are not in the health care profession. None of the anti-HBc positive participants were HBsAg positive.

KIR genotypes and genes frequencies

We next determined the *KIR* genotypes among the study participants: 44%, 40% and 16% were carriers of AA, AB and BB genotypes, respectively, with a 1.77 A to B ratio. The genotype was classified as B if any of the following genes was detected: 2DL2, 2DL5, 3DS1, 2DS1, 2DS2, 2DS3 and 2DS5. If none of these was detected, the genotype was considered as AA. Similarly, if none of the A haplotypes was detected, the genotype was classified as BB; 77%, 75% and 69% of the AA, AB and BB carriers were vaccinated, respectively. There was no significant difference between the expression of AA, AB and BB among vaccinated and non-vaccinated participants (FET, *P* = 0.784). Similarly, we did not detect any significant difference when we compared the frequencies of *KIR* genotypes among the vaccinated with anti-HBs levels less than 10, 10-100 and above 100 mIU/mL. We did not detect any difference between the frequencies of *KIR* genotypes among vaccinated participants with protective and non-protective levels of anti-HBs (FET, *P* = 0.865).

Table 2 The observed and estimated killer immunoglobulin receptor gene frequencies in the study participants

	Inhibitory *KIR*								Non-inhibitory *KIR*						Pseudogene		
	2DL1	2DL2	2DL3	2DL4	2DL5	3DL1	3DL2	3DL3	2DS1	2DS2	2DS3	2DS4	2DS5	3DS1	2DP1	3DP*001/002/004	3DP1*003
OF	99	56	84	100	42	96	100	100	40.6	55.4	46.5	95	34.7	41.6	99	17	100
KLF	0.9	0.34	0.6	1	0.24	0.8	1	1	0.23	0.33	0.27	0.78	0.19	0.24	0.9	1	1

2DL5A, 24.8%; 2DL5B, 28.7%; 2DS4*001/*002, 40.6%; 2DS4*003/007, 82.2%. KIR: Killer immunoglobulin receptor; OF: Observed frequency calculated by direct counting; KLF: Gene frequency calculated using the formula 1 - √ (1 - OF).

Table 3 Killer immunoglobulin receptor gene frequencies among study participants vaccinated against hepatitis B virus (protected and non-protected by hepatitis B vaccine) *n* (%)

KIR genes	Anti-HBsAg < 10 mIU/mL (*n* = 22)	Anti-HBsAg ⩾ 10 mIU/mL (*n* = 52)	Test[1]	OR (95%CI)	*P* value
2DL2	14 (63.60)	27 (51.90)	0.858	0.62 (0.22-1.72)	0.446
2DL3	18 (81.80)	45 (86.40)	FET	1.43 (0.37-5.48)	0.723
2DL5A	8 (36.40)	13 (25.00)	0.982	0.58 (0.20-1.70)	0.4
2DL5B	5 (22.70)	19 (36.50)	1.346	1.97 (0.62-6.16)	0.289
2DS1	11 (50.00)	19 (36.50)	1.162	0.58 (0.21-1.58)	0.31
2DS2	14 (63.60)	26 (50.00)	1.157	0.57 (0.21-1.59)	0.318
2DS3	9 (40.90)	23 (44.20)	0.069	1.15 (0.42-3.15)	0.804
2DS4*001/002	8 (36.40)	20 (38.40)	0.029	1.09 (0.39-3.072)	1.00
2DS4*003/007	19 (86.40)	41 (78.85)	FET	0.59 (0.15-2.36)	0.534
2DS5	10 (45.50)	17 (32.60)	1.087	0.58 (0.21-1.62)	0.428
3DS1	11 (50.00)	21 (40.40)	0.582	0.68 (0.25-1.85)	0.608
3DP*001/002/004	6 (27.30)	9 (17.30)	FET	0.56 (0.17-1.82)	0.355

[1]The statistical test performed to compare the frequencies of *KIR* gene expression among vaccinated and non-vaccinated study participants where by FET refers to Fisher's exact test and the rest of the values represent the Pearson χ^2 value. We compared the frequencies of *KIR* genes with enough variability between study participants that were vaccinated against hepatitis B virus. The vaccinated participants were divided for this analysis into 2 groups depending on the level of anti-HBsAg. Anti-HBsAg < 10 mIU/mL, not protected against hepatitis B virus infection; anti-HBsAg ⩾ 10 mIU/mL, protected against hepatitis B virus infection as a result of vaccination. KIR: Killer immunoglobulin receptor; FET: Fisher-exact test; HBsAg: Hepatitis B surface antigen. OR: Odds ratio.

We divided the *KIR* genes expressed in our study participants into inhibitory, non-inhibitory (or activating) and those encoding inhibitory and activating signals, as previously described[13]. The inhibitory KIR2DL1, KIR2DL4, KIR3DL1, KIR3DL2, and KIR3DL were detected in more than 95%, whereas KIR2DL2, KIR2DL3, KIR2DL5 (KR2DL5A and KIR2DL5B) were expressed in 56%, 84% and 42% (25% and 29%) of participants, respectively (Table 2). The OF of the activating *KIR* genes ranged between 35% and 55% except for KIR2DS4, detected in 95% of the study participants (40.6% 2DS4*001/002; 82.2% 2DS4*003/007). KIR2DP1 pseudogene was detected in 99% of our participants, whereas KIR3DP*001/02/04 and KIR3DP1*003 had frequencies of 17% and 100%, respectively. The corresponding estimated frequencies of each *KIR* gene followed the same trend or order as the OF data.

For this analysis and thereafter, we studied the genes with enough variability, particularly 2DL2, 2DL3, 2DL5 (2DL5A, 2DL5B), 2DS1, 2DS2, 2DS3, 2DS4 (and its variants), 2DS5, 3DS1 and the pseudogene 3DP1*001/002/004 (Table 2). We report the lack of significant difference in the frequency of *KIR* genes among vaccinated and non-vaccinated participants (Table 3). Moreover, there was no significant difference in the frequency of these genes among participants protected against HBV and those that are not protected against HBV, as judged by their level of anti-HBs antibodies (Table 4).

We performed similar analyses to determine the relationship between AA, AB, BB genotypes and the expression of *KIR* genes showing enough variability (Table 5). 2DL2 (χ^2, P = 0.00), 2DL3 (χ^2, P = 0.00), 2DS2 (χ^2, P = 0.00), 2DS3 (χ^2, P = 0.00), 2DL5B FET (P = 0.00), and 3DP1001/002/004 (FET, P = 0.006) were found to be significantly different between genotypes but not 2DS1 (χ^2, P = 0.749), 2DL5A (FET, P = 0.782), 2DS4*001/002 (χ^2, P = 0.621), 2DS4*003/007 (FET, P = 0.392) and 3DS1 (χ^2, P = 0.948). For those genes showing significant differences among the AA, AB and BB genotypes, we computed post-hoc comparisons. These differences still hold significance between subgroups except for 2DL5B, 2DS2, 2DS3 and 3DP1*001/002/004 between genotypes AB and BB.

KIR gene frequencies among vaccinated and non-vaccinated participants

To evaluate the role of *KIR* genes in protection against or susceptibility to HBV, we next compared the frequency of *KIR* genes among HBV-vaccinated and non-vaccinated participants (as judged by the level of anti-HBs antibodies). Depending on their anti-HBs levels that we tested for in this study, participants vaccinated against hepatitis B were divided into 4 groups: Group Ⅰ >

Table 4 Killer immunoglobulin receptor gene frequencies among study participants vaccinated against hepatitis B virus as compared to non-vaccinated subjects *n* (%)

KIR genes	Vaccinated (*n* = 74)	Non-vaccinated (*n* = 25)	Test[1]	OR (95%CI)	*P* value
2DL2	41 (55.40)	15 (60.00)	0.161	0.83 (0.33-2.082)	0.436
2DL3	63 (85.10)	20 (80.00)	0.364	1.43 (0.44-4.62)	0.374
2DL5A	21 (28.40)	4 (16.00)	1.517	2.08 (0.64-6.79)	0.168
2DL5B	24 (32.40)	5 (20.00)	1.395	1.92 (0.64-5.73)	0.178
2DS1	30 (40.50)	10 (40.00)	0.002	1.02 (0.41-2.58)	0.577
2DS2	40 (54.10)	15 (60.00)	0.268	0.78 (0.31-1.97)	0.39
2DS3	32 (43.20)	14 (56.00)	1.223	0.60 (0.24-1.49)	0.191
2DS4*001/002	28 (37.80)	11 (44.00)	0.297	0.77 (0.31-1.94)	0.376
2DS4*003/007	60 (81.10)	22 (88.00)	FET	0.58 (0.15-2.23)	0.324
2DS5	27 (36.50)	7 (28.00)	0.597	1.47 (0.55-3.99)	0.302
3DS1	32 (43.20)	9 (36.00)	0.246	1.35 (0.53-3.46)	0.401
3DP*001/002/004	15 (20.30)	2 (8.00)	FET	2.92 (1.62-13.80)	0.134

We compared the frequencies of *KIR* genes with enough variability between study participants that were vaccinated against hepatitis B virus and those that were not. [1]The statistical test performed to compare the frequencies of *KIR* gene expression among vaccinated and non-vaccinated study participants whereby FET refers to Fisher's exact test and the rest of the values represent the Pearson χ^2 value. KIR: Killer immunoglobulin receptor; FET: Fisher-exact test; OR: Odds ratio.

Table 5 The relationship between AA, AB and BB genotypes and the expression of killer immunoglobulin receptor genes among the study participants *n* (%)

KIR genes	AA (*n* = 44)	AB (*n* = 41)	BB (*n* = 16)	Test[1]	*P* value
2DL2	0 (0.00)	41 (100.00)	16 (100.00)	101	0.00[a]
2DL3	44 (100.00)	41 (100.00)	0 (0.00)	101	0.00[a]
2DL5A	10 (22.70)	10 (22.70)	5 (31.25)	FET	0.782
2DL5B	1 (2.27)	20 (24.39)	8 (50.00)	FET	0.00[a]
2DS1	16 (36.30)	18 (43.90)	7 (43.75)	0.579	0.749
2DS2	0 (0.00)	40 (97.50)	16 (100.00)	97.051	0.00[a]
2DS3	3 (6.80)	30 (73.17)	14 (87.50)	50.38	0.00[a]
2DS4*001/002	16 (36.40)	19 (37.50)	6 (37.50)	0.952	0.621
2DS4*003/007	38 (86.40)	31 (75.60)	14 (87.50)	FET	0.392
2DS5	14 (31.80)	16 (39.00)	5 (31.30)	0.584	0.747
3DS1	18 (40.90)	18 (43.90)	6 (40.00)	0.107	0.948
3DP*001/002/004	2 (4.50)	10 (24.40)	5 (31.30)	FET	0.0006[a]

[1]The statistical test performed to determine the relationship between AA, AB and BB genotypes and the expression of *KIR* genes among vaccinated and non-vaccinated study participants. FET refers to the Fisher's exact test and the rest of the values represent the Pearson χ^2 value. [a]$P < 0.05$, significant. KIR: Killer immunoglobulin receptor; FET: Fisher-exact test.

100 mIU/mL, group Ⅱ 10-100 mIU/mL, group Ⅲ < 10 mIU/mL and group Ⅳ with undetectable levels of protective antibodies. The frequency of only KIR2DL5B was significantly different among these categories (FET, $P = 0.0263$) (Table 6). When we performed post-hoc comparisons between these groups, we detected no significant difference in the expression of KIR2DL5B. These groups of vaccinated participants were also similar in relation to the expression of AA, AB and BB genotypes (FET, $P = 0.669$).

We identified 3 participants testing positive for anti-HBc with anti-HBs levels higher than 100 mIU/mL. Two out of three of these participants were non-vaccinated and thus we believe they are protected as a result of natural infection. The third participant was vaccinated against HBV. Two out of three (66.7%) of these participants carry the AB genotype and one participant is AA positive. The three participants expressed 2DL1, 2DL3, 3DL1, 3DL2, 3DL3 (inhibitory), activating genes (2DS3, 2DS4*001/002 variant) and both inhibitory and activating genes (2DL4, 2DP1 and 3DP1*003). These subjects did not express 2DL5A or 3DP1*001/002/004. We did not test for the presence of HBV DNA among these participants. We did not find any significant relationship between the genotype of these participants and the expression of *KIR* genes, or between the *KIR* genes and the susceptibility to natural or breakthrough infection.

DISCUSSION

The administration of hepatitis B vaccine in infancy is 95% effective and correlates with long-term protection[8,35,36]. However, vaccine failure has been reported in 5% of hepatitis B-vaccinated persons; moreover, breakthrough infection has also been reported following vaccination with hepatitis B vaccine[37]. The increase

Table 6 Killer immunoglobulin receptor gene expression and levels of anti-HBs among the vaccinated study participants *n* (%)

KIR genes	Group Ⅰ[1] (*n* = 36)	Group Ⅱ[2] (*n* = 16)	Group Ⅲ[3] (*n* = 15)	Group Ⅳ[4] (*n* = 7)	Test[5]	*P* value
2DL2	17 (47.20)	10 (62.50)	11 (73.30)	3 (42.90)	FET	0.362
2DL3	31 (86.10)	14 (87.50)	12 (80.00)	6 (85.70)	FET	0.824
2DL5A	9 (25.00)	4 (25.00)	4 (26.70)	4 (57.10)	FET	0.987
2DL5B	9 (25.00)	10 (62.50)	4 (26.70)	1 (14.30)	FET	0.023[6]
2DS1	11 (30.60)	8 (50.00)	7 (46.70)	4 (57.10)	FET	0.530
2DS2	16 (44.40)	10 (62.50)	11 (73.30)	3 (42.90)	FET	0.270
2DS3	12 (33.30)	11 (68.80)	8 (53.30)	1 (14.30)	5.01	0.060
2DS4*001/002	15 (41.70)	5 (31.30)	6 (40.00)	2 (28.60)	0.568	0.920
2DS4*003/007	28 (77.80)	13 (81.30)	13 (86.70)	6 (85.70)	FET	0.860
2DS5	11 (30.60)	6 (37.50)	6 (40.00)	3 (42.90)	0.846	0.850
3DS1	14 (38.90)	7 (43.80)	7 (46.70)	6 (85.70)	FET	0.960
2DL4	36 (100.00)	16 (100.00)	15 (100.00)	4 (57.10)	0.258	0.970
3DP*001/002/004	8 (22.20)	1 (6.30)	4 (26.70)	1 (14.30)	FET	0.420

[1]Group Ⅰ: Vaccinated participants and anti-HBs titers > 100 mIU/mL; [2]Group Ⅱ: Vaccinated participants and anti-HBs titers 10-100 mIU/mL; [3]Group Ⅲ: Vaccinated participants and anti-HBs titers 0.1-9.99 mIU/mL; [4]Group Ⅳ: Vaccinated participants and anti-HBs titers = 0 mIU/mL; [5]The statistical test performed to compare the frequencies of *KIR* gene expression among the vaccinated study participants whereby FET refers to Fisher's exact test and the rest of the values represent the Pearson χ^2 value; [6]Significant difference of KIR2DL5B expression among vaccinated study participants ($P < 0.05$). KIR: Killer immunoglobulin receptor; FET: Fisher-exact test.

in circulating NK cells, major players in the innate immune system and regulators of the virus-specific T cell responses through their cross-talk with dendritic cells and T cells[38-40], was suggested to contribute to HBV viral control[41]. The impact of genetic regulation on immune responses following vaccinations has been previously reported[19,24]. This evidence prompted us to explore the potential role of KIR following hepatitis B vaccination. In Lebanon, hepatitis B vaccine is offered as part of the immunization program early in childhood as per the WHO guidelines. In this study, 69% of the vaccinated participants retained more than 10 mIU/mL of anti-HBs antibodies and hence are immune to HBV infection; whereas 30% are susceptible to the latter due to either undetectable levels of antibodies or levels below 10 mIU/mL. We do not have data on the time of vaccination of these participants to reflect on the duration of the retention or the loss of the immune response post-vaccination. We report the lack of significant association between the number of vaccine dosages (when vaccine dosage is available) and the titer of antibodies among vaccinated participants. Recent reports show that multiple immunizations against hepatitis B are inefficient at mounting antibody responses[42], while others suggest that immunization against hepatitis in infancy is associated with a seroprotective response to a challenge dose of vaccine with extended duration of protection through adolescent years[43]. We cannot suggest similar trends from our results due to the lack of data on the time of vaccination, the age at vaccination, as well as the number of dosages administered for many participants.

Anti-HBc antibodies, indicators of HBV infection, were detected in 3 participants (3%) in the absence of HBsAg, with one being a nurse suspected of being exposed to HBV at the work place. This might suggest a "breakthrough" infection occurring following vaccination against hepatitis B; this is suggested since health care workers are regularly monitored for protective levels of anti-HBs antibodies. However, due to the lack of data on the timing of vaccination and/or infection of this participant, we cannot confirm whether exposure to HBV has occurred before or after vaccination. The other 2 participants are non-vaccinated and are protected with high levels of anti-HBs antibodies as a result of natural infection. We do not have data pertaining to the time of infection following vaccination; moreover, we did not perform HBV DNA testing. Health care workers with undetectable anti-HBsAg levels detected in our study are clearly susceptible to HBV infection and consequently in need of booster vaccination to induce an anamnestic response in order to prevent acute disease and carrier state.

While hepatitis B vaccine booster doses are not currently recommended following vaccination, a better understanding of the correlates of long-term immunity is needed. This is critical especially since several studies show that vaccines with anti-HBs levels of 10-99 mIU/mL achieved following primary vaccination are less likely to produce an anamnestic response following a booster HBV vaccine as compared to those with anti-HBs $\geqslant$ 100 mIU/mL[35,44]. NK cells play a major role in the innate immune system as first line of defense and in the regulation of the virus-specific T cell responses through their cross-talk with dendritic cells and T cells[38-40]. Moreover, NK cells are suggested to contribute to HBV control[41]. Our data show that genotypes with 11 *KIR* genes were most prevalent, with AA genotype being more frequent among the study participants. The inhibitory *KIR* genes were more frequent among our study participants than the activating genes, which is in agreement with a finding associated with A haplotype being present in higher numbers in inhibitory *KIR* genes[39].

KIR2DL4, KIR3DL2, KIR3DL3 and KIR3DP1*003 were present in every participant. This is expected since these

are framework genes. The frequency of KIR2DL5B is the only significantly different gene among the vaccinated participants with different anti-HBs antibodies titer. The role of KIR2DL5, expressed at frequencies ranging between 26% and 86% in all human populations, is not completely understood[45]. The ligand of KIR2DL5 is also still unknown.

A number of limitations exist, and these include the lack of data on the time of vaccination and corresponding age at time of vaccination of the study participants, and more importantly, the small sample size. Our sample size is powered to detect medium to large effect sizes when some of the effect sizes for group differences are small. However, medium to large effect sizes are those where group differences have more clinical significance, which we are powered to detect. Consequently, the clear impact that *KIR* genes have on susceptibility to acquiring hepatitis B or protection against the infection cannot be addressed in these small groups.

To our knowledge, this is the first study screening for the possible role of *KIR* genes among individuals vaccinated against HBV. While studies have shown the association between gene variants and immune responses to a variety of vaccines, little is known about the strength and the sustainability of antibody responses following vaccination against HBV in relation to expression of *KIR* genes. Our results are useful to design larger studies to better elucidate the role of KIR in susceptibility or long-term protection against HBV as well as other diseases.

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COMMENTS

Background

Killer immunoglobulin receptor (*KIR*) genes encode receptors expressed on the surface of natural killer cells. The literature has described the relationship between KIRs and differential responses in many disease conditions, specifically human immunodeficiency virus and hepatitis C virus. The authors thought to explore the role of *KIR* genes in response to hepatitis B vaccine in a cohort of Lebanese adults.

Research frontiers

This study aims at elucidating the possible role of genetic factors such as KIRs in the regulation of post-vaccine immune responses specifically following hepatitis B vaccine.

Applications

While the sample size is powered to detect medium to large effect sizes, the impact that KIR and HLA have on the susceptibility to acquiring hepatitis B virus (HBV) or protection against the infection cannot be addressed in our sample. Nevertheless, our results are useful to design larger studies to better elucidate the role of KIR in susceptibility or long-term protection against HBV and other diseases.

Terminology

Two groups of *KIR* haplotypes are known in humans: A and B. Haplotype A encodes inhibitory receptors and consists of nine genes [3DL3, 2DL3, 2DP1, 2DL1, 3DP1, 2DL4, 3DL1, one activating (2DS4), 3DL2 and 2DL5]. Haplotype B carries a variety of gene combinations and encodes more activating receptors as compared to haplotype A [3DL3, 2DS2, 2DL2, 2DL5B (inhibitory), 2DS3, 2DP1, 2DL1, 3DP1, 2DL4, 3DS1, 2DL5A (inhibitory), 2DS5, 2DS1, and 3DL2].

Peer-review

This is an interesting study aiming to screen for a possible role of *KIR* gene expression and antibody response following hepatitis B vaccination. The major point of this manuscript is that there is no significant association between the frequency of *KIR* genes and anti-HBs antibodies detected. Although it is a negative result, it could be an indicant for understanding the role of *KIR* loci in response to HB vaccine.

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ORIGINAL ARTICLE

Basic Study

Lycopene modulates cellular proliferation, glycolysis and hepatic ultrastructure during hepatocellular carcinoma

Prachi Gupta, Nisha Bhatia, Mohinder Pal Bansal, Ashwani Koul

Prachi Gupta, Nisha Bhatia, Mohinder Pal Bansal, Ashwani Koul, Department of Biophysics, Basic Medical Sciences Block Ⅱ, Panjab University, Chandigarh 160014, India

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Correspondence to: Ashwani Koul, Professor, Department of Biophysics, Basic Medical Sciences Block Ⅱ, Panjab University, South Campus, Sec-25, Chandigarh 160014, India. drashwanikoul@yahoo.co.in
Telephone: +91-172-2534119

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Abstract

AIM

To investigate the effect of lycopene extracted from tomatoes (LycT) on ultrastructure, glycolytic enzymes, cell proliferation markers and hypoxia during N-Nitrosodiethylamine (NDEA)-induced hepatocarcinogenesis.

METHODS

Female BALB/c mice were randomly divided into four groups: The Control, NDEA (200 mg NDEA/kg b.w. given i.p.), LycT (5 mg/kg b.w. given orally on alternate days) and LycT + NDEA group. The mRNA and protein expression of various cell proliferation markers (PCNA, Cyclin D1, and p21) were assessed by reverse transcription-polymerase chain reaction and enzyme linked immunosorbent assay, respectively. The ultrastructure of hepatic tissue was analyzed using scanning and transmission electron microscopy. The enzymatic activity of glycolytic enzymes was estimated using standardized protocols, while glucose-6-phosphate dehydrogenase activity level was estimated using a kit obtained from Reckon Diagnostic P. Ltd. (India).

RESULTS

Uncontrolled proliferation in the liver of NDEA ($P \leqslant 0.001$) mice was evident from the high expression of cell-proliferation associated genes (PCNA, Cyclin D1, and p21) when compared to control and LycT mice. In addition, enhanced activities of hexokinase, phosphoglucoisomerase, aldolase, glucose-6-phosphate

dehydrogenase and hypoxia-inducible factor-1α were observed in NDEA mice as compared to control ($P \leqslant 0.001$) and LycT ($P \leqslant 0.001$) mice. The alterations in hepatic ultrastructure observed in the NDEA group correlated with the changes in the above parameters. LycT pre-treatment in NDEA-challenged mice ameliorated the investigated pathways disrupted by NDEA treatment. Moreover, hepatic electron micrographs from the LycT + NDEA group showed increased macrophages, apoptotic bodies and well-differentiated hepatocellular carcinoma (HCC) in comparison to undifferentiated HCC as observed in the NDEA treated group.

CONCLUSION

This study demonstrates that dietary supplementation with LycT has a multidimensional role in preventing HCC development.

Key words: Hepatocellular carcinoma; Ultrastructure; Hypoxia; Cell proliferation; Lycopene; Glycolysis

Core tip: The present study was designed to evaluate the chemopreventive role of lycopene extracted from tomatoes (LycT) against N-Nitrosodiethylamine-induced hepatocellular carcinoma (HCC). The findings suggested the mechanism underlying LycT-mediated chemoprevention of HCC.

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INTRODUCTION

The continuous rising trend in cancer worldwide necessitates potential action against this deadly disease. Cancer is a complex disease and requires attention in multiple directions to prevent its development. Nearly two-thirds of all cancer cases are linked to inadequate components in the diet, environmental exposure to pollutants and occupational exposure to toxic materials[1,2]. Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and is the second most common cause of cancer-related death[3]. N-Nitrosodiethylamine (NDEA) is a known potent environmental hepatic carcinogen and has been used as an initiator in several hepatic cancer models[4,5]. Besides direct exogenous exposure, humans are also exposed to endogenously produced nitrosamines[6]. NDEA is metabolized in the liver to its active ethyl radical metabolite and various other reactive metabolites, which are highly reactive towards DNA, proteins and lipids, thus exhibiting sequential cellular and molecular alterations leading to its hepatocarcinogenic effect[4,7-9].

Natural and experimental chemical hepatocarcinogenesis is accompanied by altered cellular redox status, altered cytochemical pathways, altered molecular phenomena, chromosomal instability and altered physiological environment in cells. Of the various carcinogenic insults, uncontrolled proliferation, dysregulated carbohydrate metabolism and hypoxia play very crucial roles in HCC development. Aerobic glycolysis and diversion to a biosynthetic pathway, *i.e.,* the pentose phosphate pathway are the metabolic hallmarks of carcinogenesis[10]. In recent years, interest in these pathways has been renewed in the tumor microenvironment which has a profound effect on core tumor metabolism. Hypoxia inducible factor-1α (HIF-1α) is involved in many compensatory pathways such as angiogenesis, glucose metabolism, survival and tumor development[11]. Although there have been advances in therapeutic approaches a complete cure is still unavailable. Dietary multi-targeted agents have attracted the attention of cancer biologists as these agents may provide a solution to this complex problem by targeting multiple targets simultaneously[12]. A large body of evidence has revealed an association between phytochemicals and a reduced risk of developing chronic diseases[13,14].

Lycopene, a polyunsaturated hydrocarbon imparting red colour to various fruits is a nutritionally important carotenoid exhibiting beneficial health effects by virtue of its antioxidant activity with minimal side effects[15]. A large number of studies have shown an association between lycopene and a reduced risk of developing chronic diseases such as cancer, diabetes, cardiovascular disorders and degenerative diseases[15-18]. In addition to its antioxidant property, lycopene is known to modulate other non-oxidative pathways such as regulation of gap junction communication, the hormonal system, the immune system and the metabolic pathways of xenobiotics[16,19]. Phytochemicals also tend to be more effective for long-standing health problems that do not respond well to synthetic medicines[20].

In our previous studies we developed a standardized detailed protocol for lycopene extracted from tomatoes (LycT), its characterization and its beneficial effect in inhibiting NDEA-induced HCC development in terms of histopathological observations, tumor statistics, apoptosis, antioxidative capacity and toxicity[21-23]. However, further studies are warranted to determine the modulating effect of lycopene on dysregulated glucose metabolism and hypoxia, as these processes play critical roles in cancer. Thus, the present study was designed to explore the influence of LycT on various glycolytic and non-glycolytic enzymes, the expression of *HIF-1α* and potent cell proliferation-associated genes, while preventing NDEA-induced HCC. Moreover, an attempt was made to demonstrate the impact of an imbalance between energy production and metabolic demands on the gross morphology and ultrastructure of hepatocytes in HCC.

MATERIALS AND METHODS

Chemicals

Azino-bis(ethylbenzthiazoline sulfonic acid) (ABTS),

diaminobenzidine, ethidium bromide, TRI-reagent and NDEA were obtained from Sigma Chemicals (St. Louis, MO, United States). Primary and secondary antibodies were obtained from Santa Cruz Biotechnology, CA, United States. Invitrogen superscript (Ⅲ) one step reverse transcription-polymerase chain reaction (RT-PCR) was purchased and used for RT-PCR analysis. Other chemicals were purchased from local reputable companies including Sisco Research Laboratory (P) Ltd. Detailed information regarding extraction and characterization of LycT has been reported previously (Gupta *et al*[21]).

Animal model and experimental conditions

The animal protocol was designed to minimize pain or discomfort to the animals. All animals were acclimatized to laboratory conditions, *i.e.,* temperature of 21 ℃ ± 1 ℃ and humidity of 50%-60% for one week prior to the various treatments. All the mice were provided with drinking water and a standard animal pellet diet ad libitum. Female BALB/c mice (25-30 g) were randomly divided into four groups (n = 7 per group). Animals in Group Ⅰ (Control) received 0.1 mL olive oil (vehicle) orally throughout the experiment. Group Ⅱ (NDEA) animals received a cumulative dose of 200 mg NDEA/kg body weight (b.w.) given intraperitoneally in 8 wk as described previously[5]. Group Ⅲ (LycT) mice received LycT orally at a dose of 5 mg/kg b.w. thrice a week for 24 wk. Group Ⅳ (LycT + NDEA) animals received NDEA in the same manner as Group Ⅲ and were also given LycT at a dose of 5 mg/kg b.w. thrice a week for 24 wk. LycT administration was commenced two weeks prior to NDEA treatment. Animals were sacrificed after the 24th week to evaluate the modulatory effect of LycT in NDEA-induced hepatocarcinogenesis. The experimental study was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of Panjab University, Chandigarh (India) and conducted according to the Indian National Science Academy guidelines for the use and care of experimental animals (IAEC/284-295 at Sr. No. 48).

Scanning and transmission electron microscopy

After 24 wk, liver tissues from animals in the different groups were immediately fixed in 2% para-formaldehyde and 2.5% glutaraldehyde prepared in 100 mmol/L phosphate buffer (pH 7.4) for 6 h at 4 ℃. Critical point drying, trimming of the tissue and gold coating were carried out and the tissues were viewed under a LEO 435 VP scanning electron microscope. Secondary fixation, treatment with a mixture of propylene oxide and epoxy resin (1:1), embedding in freshly prepared epoxy resin and ultrathin sections mounted on colloid on-carbon coated grids were carried out and examined with a Philips CM-10 transmission electron microscope.

Estimation of the activities of glycolytic enzymes, glucose-6-phosphate dehydrogenase and glycogen content

The specific activity of hexokinase, phosphoglucoisomerase (PGI) and aldolase was estimated according to the reported standard protocols[24-26]. Glucose-6-phosphate dehydrogenase (G6PD) activity level was estimated in a tissue homogenate using an ENZOPAK G6PD kit obtained from Reckon Diagnostic P. Ltd. (India). The activity of G6PD in the samples was further calculated using 6.22 mmol/L per centimeter as the extinction coefficient of NADPH at 340 nm. The glycogen content in liver was estimated using the protocol described by Seifter *et al*[27]. The amount of glycogen in the aliquot was determined using a glucose standard.

mRNA expression analysis

Total RNA isolation from liver tissue was carried out using TRI-reagent. For RT-PCR analysis, primers for *HIF-1α, PCNA, p21* and *Cyclin D1* were searched from the database "Gene Runner" and were synthesized by Sigma-Aldrich (United States). The lengths of the primers chosen were approximately 20bp (Table 1). RT-PCR was performed according to the described protocol of the Superscript (Ⅲ) one step RT-PCR kit. The DNA bands were visualized in agarose gel using an ultraviolet transilluminator and photographed on Gel Doc. Densitometric analysis of the bands was performed using Image J software (National Institute of Health, United States).

Quantitation of protein expression

Sample preparation: The animals were fasted overnight before liver dissection. Mice were euthanized by cervical dislocation under light ether anesthesia. Liver perfusion was carried out with 0.9% NaCl and the liver was carefully removed and placed in a Petri plate containing ice-cold saline. The tissue was homogenized in ice-cold 100 mmol/L potassium phosphate buffer (pH 7.4) containing 150 mmol/L KCl in an ice-chamber to obtain 25% homogenate (w/v) using a mechanically driven Teflon fitted Potter Elveihem homogenizer. The homogenate (25%) was then subjected to centrifugation at 10000 rpm for 30 min at 4 ℃ for preparation of the post-mitochondrial fraction.

ELISA: Post mitochondrial fractions obtained from the hepatic tissue of different groups were quantitated for protein concentration by the method of Lowry *et al*[28]. Two point five microgram protein was loaded onto an ELISA strip containing carbonate buffer. Further, protein expression of *HIF-1α, PCNA, p21* and *Cyclin D1* were analyzed according to the standard protocol of ELISA using specific primary antibodies and enzyme conjugated secondary antibodies. ABTS in citrate buffer was added along with hydrogen peroxide for color generation. The color thus obtained was quantified at 405 nm.

Statistical analysis

The statistical methods used in this study were reviewed by Dr. Neha Arora Chugh, Department of Biophysics, Panjab University, Chandigarh. Data were expressed as mean ± SD. The results were subjected to analysis of variance (one-way ANOVA) followed by the post

Table 1 List of primer pairs used

Gene	Strand	Primer
HIF-1α	Sense	5′-GGT/CAG/ATG/ATC/AGA/GTC/C-3′
	Antisense	5′-TGC/TTG/GTG/CTG/ATT/TGTG/A-3′
PCNA	Sense	5′-GAT/GTG/GAG/CAA/CTT/GGA/AT-3′
	Antisense	5′-AGC/TCT/CCA/ACT/TGC/AGA/AAA-3′
p21	Sense	5′-CCG/TGG/ACA/GTG/AGC/AGT/TG -3′
	Antisense	5′-TGG/GCA/CTT/CAG/GGT/TTT/CT-3′
Cyclin D1	Sense	5′-CAC/AAC/GCA/CTT/TCT/TTC/CA-3′
	Antisense	5′-GAC/CAG/CCT/CTT/CCT/CCA/C-3′
β-actin	Sense	5′-ATC/CGT/AAA/GAC/CTC/TAT/GC-3′
	Antisense	5′-AAC/GCA/GCT/CAG/TAA/CAG/TC-3′

hoc test for statistical significance using SPSS (version 14.0) software. $P \leqslant 0.05$ was considered statistically significant.

RESULTS

Scanning electron microscopy (SEM) of the control and LycT groups revealed normal hepatic surface morphology with polyhedral hepatocytes radially arranged around central veins in cords separated by sinusoids (Figure 1A-C). Bile canaliculi were observed on the apical surface of hepatocytes. A few red blood cells were also visible in the sinusoids. However, serious and irreversible alterations in liver architecture were observed in the NDEA and LycT + NDEA groups. Smoothing or rounding of the hepatocytes with hyperplastic tumor along with clumps of hepatocytes with intercellular surfaces covered with numerous microvillus projections were visible (Figure 1D-E). The discernible nodules were of irregular shape and size. Necrotic tumor nodules and uncontrolled cell density revealed the presence of undifferentiated HCC in the NDEA group. In contrast, the surface morphology of liver tissue from the LycT + NDEA group revealed well differentiated HCC characterized by stromal invasion and a trabecular pattern of two to three cells thick plates of hepatocytes (Figure 1H). High cell density with pleomorphism of tumor cells was also evident (Figure 1F). Apoptotic bodies were observed indicating a high rate of apoptosis (Figure 1G). Thus, LycT pre-treatment of NDEA-challenged mice significantly reduced the severity caused by NDEA. Table 2 shows the results of a quantitative comparison of hepatic tissues from the NDEA and LycT + NDEA groups using SEM.

Transmission electron microscopy (TEM) of the control and LycT groups revealed normal hepatic ultrastructural architecture (Figure 2A-C). At low magnification, hexagonal hepatocytes radially arranged around blood vessels with an intact cell membrane, clear and granulated cytoplasm comprising oval-shaped mitochondria, rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum were observed. A smooth, rounded and prominent nucleus with intact double layered nuclear membrane, a darkly stained single and prominent nucleolus along with uniformly distributed chromatin in the nucleoplasm

Table 2 Comparative analysis of the N-Nitrosodiethylamine and lycopene extracted from tomatoes + N-Nitrosodiethylamine group by scanning electron microscopy

Groups/parameters	NDEA	LycT + NDEA
Cell density	+++	+
Rounding of hepatocytes	+++	+
Trabecular structures	-	+++
Necrotic tumor nodules	+++	+
Apoptotic bodies	-	+++
Type of HCC	Undifferentiated	Well-differentiated

Where "+++" indicates that more than 70%-90% of mice in a group showed this feature; "+" indicates that < 70% of mice showed this feature; "-" indicates that the < 20% of mice showed this feature. LycT: Lycopene extracted from tomatoes; NDEA: N-Nitrosodiethylamine; HCC: Hepatocellular carcinoma.

were also visible. Irregular non-membrane bound, faintly stained granules of hepatocellular glycogen were also observed. The presence of lipid granules as darkly stained spots and a few bi-nucleated hepatocytes were found in the LycT group in addition to the above features. Several irreversible alterations in the nucleus of liver cells from the NDEA group were observed (Figure 2D-E). A prominent large and irregular nucleus with interrupted nuclear membrane, multiple prominent nuclei and pseudo-inclusions were observed in liver sections from the NDEA group with an increased nuclear/cytoplasmic ratio. Karyotin (reticular material) deposition along the nuclear membrane was the striking difference when compared with normal nuclei. Loss of organization of cytoplasmic components such as pleomorphic mitochondria varying in shape and size, dilated cisternae of RER associated with mitochondria and decreased lysosomes were observed in tumor cells from the NDEA group. Variable light and dark granules of fat and glycogen deposits were also observed. The liver cells from the LycT + NDEA group showed mild damage to the nuclear membrane with fewer karyotin deposits (Figure 2F). Hepatocytes were evident with prominent nucleoli, pleomorphic mitochondria and a higher number of lysosomes. Moreover, liver sections showed many macrophages in sinusoids and apoptotic cells (Figure 2G-H). Table 3 shows the results of a quantitative comparison of hepatic tissues from the NDEA and LycT + NDEA groups using TEM.

A significant increase in the activities of liver hexokinase, PGI and aldolase in the NDEA and LycT + NDEA groups was evident when compared to the control and LycT groups. However, LycT pre-treatment significantly lowered the activity of hexokinase and PGI in the LycT + NDEA group when compared to the NDEA group. No significant change in aldolase level was observed in the LycT + NDEA group when compared to the NDEA group (Figure 3A-C). Moreover, NDEA treatment caused a significant increase in liver G6PD activity in the NDEA group when compared to the control and LycT groups. Moreover, a significant increase was also observed in the levels of liver G6PD in the LycT + NDEA group when compared to the control group. However, G6PD level

Table 3 Comparative analysis of the N-Nitrosodiethylamine and lycopene extracted from tomatoes + N-Nitrosodiethylamine group by transmission electron microscopy

Groups/parameters	NDEA	LycT + NDEA
Hepatocytes	Rounded, smaller in size	Polygonal but rounded edges
Nucleus	Large and irregular shape	Oval shaped
Nuclear membrane	Not uniform and pseudoinclusions	Not uniform
Nucleoli	Large, irregular, multiple	One-two
Nuclear/cytoplasmic ratio	High	Low
Karyotin (reticular material)	Deposition along nuclear membrane	Fewer karyotin deposits
Cytoplasm	Loss of organization, dense	Organized
Mitochondria	Pleomorphic with increased density	Pleomorphic but number less than that in the NDEA group
Lysosomes	Few in number	High in number
Fat and glycogen globules	Variable	High
Macrophages	Very few	Many
Apoptotic bodies	No	Clearly visible in the section

LycT: Lycopene extracted from tomatoes; NDEA: N-Nitrosodiethylamine.

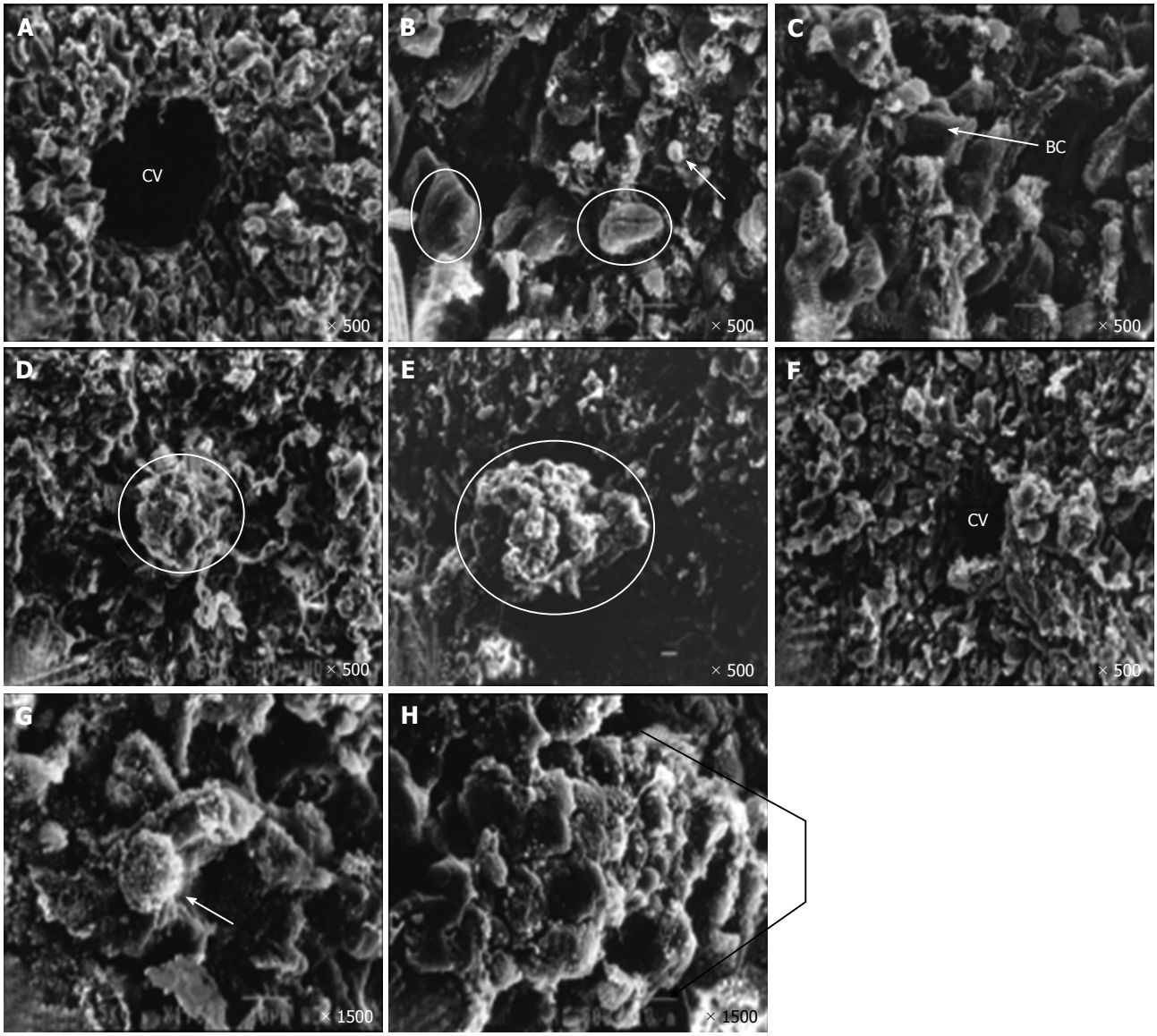


Figure 1 Scanning electron micrographs of liver tissue at × 500 and × 1500. A and B: Control group, illustrating the central vein (CV), hexagonal hepatocytes (encircled) and biconcave RBCs in sinusoids (arrowed); C: LycT group illustrating hexagonal hepatocytes with bile canaliculi (BC) on theits surface (arrowed); D and E: NDEA group illustrating tumor nodules (encircled) with abnormal cell proliferation and disturbed ultrastructure; F-H: LycT + NDEA group respectively illustrating high cell density, CV, apoptotic bodies (arrowed) along with various alterations and two-three cell plate thickening indicating well-differentiated hepatocellular carcinoma, respectively. LycT: Lycopene extracted from tomatoes; NDEA: N-Nitrosodiethylamine; RBCs: Red blood cells.

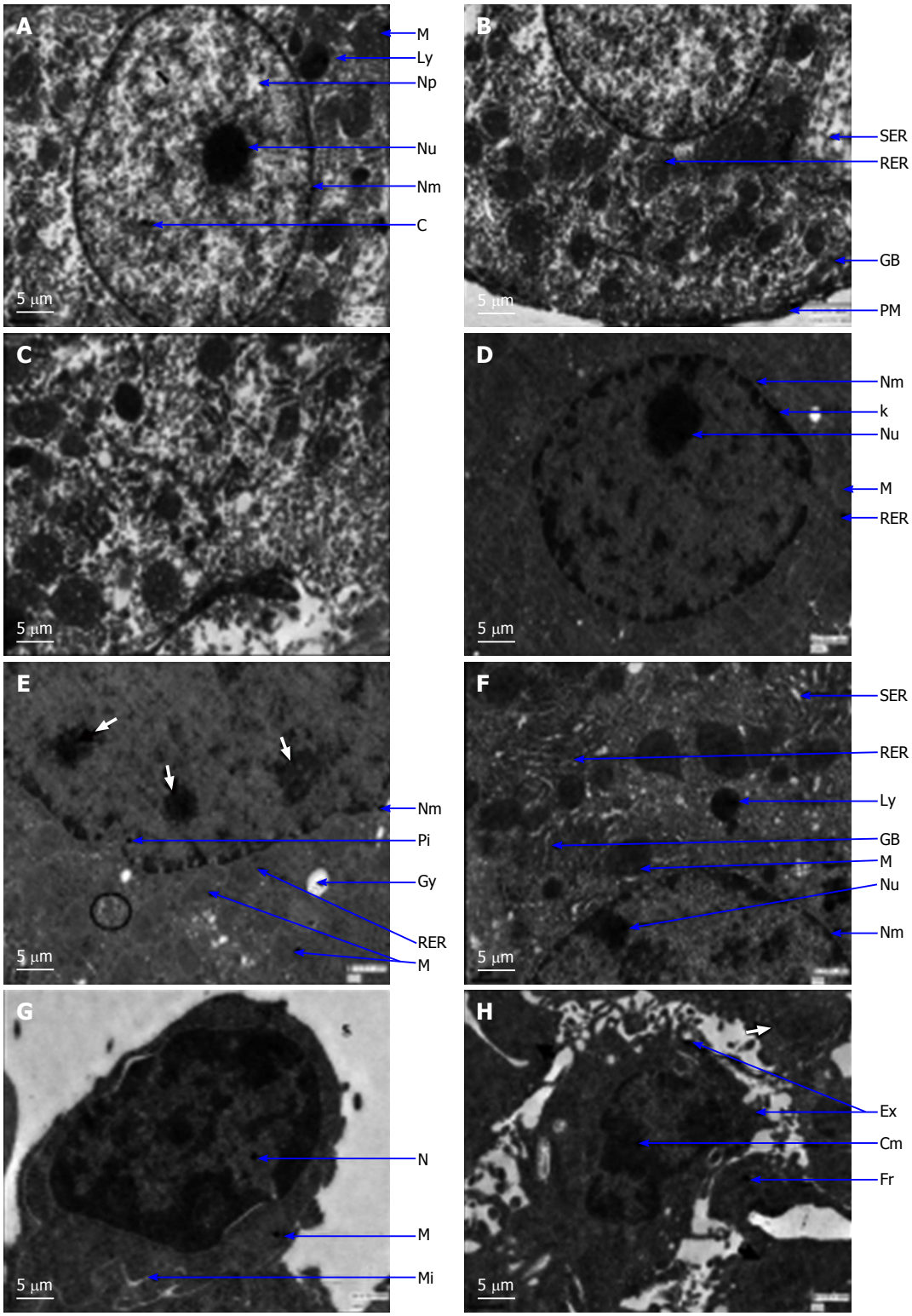


Figure 2 Transmission electron micrographs (× 2550) of liver tissue. A and B: Control group illustrating round nucleus (N), nuclear membrane (Nm), nucleolus (Nu), and chromatin (C) and nucleoplasm (Np). Clear cytoplasm with mitochondria (M), dark bodies as lysosomes (Ly), smooth endoplasmic reticulum (SER), rough endoplasmic reticulum (RER), and Ggolgi bodies (GB) surrounded by plasma membrane (PM) were observed; C: LycT group illustrating similar characteristics to those in theof control liver micrograph; D and E: NDEA group illustrating deformed N with disrupted and convoluted Nm, darkly masses in the nucleolus (white arrows), deposition of karyotin (k) and pseudo-inclusions (Pi). Cytoplasm was also found to be compactly packed with multiple pleomorphic M and RER. Percentage of other organelles was found to be lower, and variable light and dark granules of fat (encircled) and glycogen (Gy) deposits were also observed; F-H: LycT + NDEA group illustrating perforated nuclear membrane. Cytoplasm containedwas occupied with pleomorphic mitochondria, with ahowever higher percentage of organelles such as GB, Ly, RER and higher glycogen deposits. Macrophages in the sinusoid (S) were characterized by a large nucleus to cytoplasmic ratio, abundant mitochondria and microvilli (Mi). Apoptotic body characterized by cell shrinkage and condensation of nuclear chromatin into delineated masses (Cm), forming extensions (Ex), and crowded with closely packed cellular organelles (white arrow) and fragments of nucleus (Fr). LycT: Lycopene extracted from tomatoes; NDEA: N-Nitrosodiethylamine.

Table 4 Effect of N-Nitrosodiethylamine and/or lycopene extracted from tomatoes on protein expression in mice hepatic tissue using ELISA

Protein (absorbance at 405 nm)	Control	NDEA	LycT	LycT + NDEA
PCNA	0.31 ± 0.01	0.47 ± 0.01[a]	0.31 ± 0.01[b]	0.39 ± 0.01[a,b,c]
Cyclin D1	0.25 ± 0.02	0.38 ± 0.02[a]	0.24 ± 0.02[b]	0.36 ± 0.01[a,c]
p21	0.40 ±0.02	0.33 ± 0.02[e]	0.39 ± 0.02[f]	0.37 ± 0.01[g]
HIF-1α	0.22 ± 0.03	0.41 ± 0.01[a]	0.23 ± 0.03[b]	0.36 ± 0.04[a,c]

[a]$P \leqslant 0.05$, compared to the NDEA group; [b,c,e]$P \leqslant 0.001$, compared to the control group, NDEA group and LycT group, respectively; [f,g]$P \leqslant 0.01$, compared to the control group and NDEA group, respectively. LycT: Lycopene extracted from tomatoes; NDEA: N-Nitrosodiethylamine; PCNA: Proliferating cell nuclear antigen; p21: Cyclin-dependent kinase inhibitor 1A; HIF-1α: Hypoxia inducible factor-1α.

following LycT pre-treatment in NDEA-challenged mice was observed to be significantly lower than that in the NDEA group. No significant change was observed in the level of G6PD in the LycT group when compared to the control group (Figure 3D). A significant decrease in liver glycogen level was observed in the NDEA group when compared to the control and LycT groups. LycT pre-treatment in NDEA-challenged mice caused a significant increase in the levels of tissue glycogen when compared to the NDEA group. However, a significant decrease in tissue glycogen level was observed when compared to the control and LycT groups (Figure 3E). No significant change was observed in the activities of these enzymes and the level of glycogen in the LycT group when compared to the control group.

Figure 4 shows the mRNA expression of various genes involved in proliferation during HCC in the different treatment groups. Densitometric analysis of *HIF-1α* expression revealed a significant increase in the NDEA and LycT + NDEA groups when compared to the control and LycT groups (Figure 4). A significant increase in the expression of *PCNA* and *Cyclin D1* was observed in the NDEA group when compared to the control and LycT groups. The LycT + NDEA group showed a significant decrease in mRNA expression of *PCNA* and *Cyclin D1* when compared to the NDEA group. A significant increase in the expression of *Cyclin D1* was observed in the LycT + NDEA group when compared to the control and LycT groups. Densitometric analysis of *p21* expression revealed a significant decrease in the NDEA group when compared to the control and LycT groups. A significant increase in the expression of *p21* was observed in the LycT + NDEA group when compared to the control, NDEA and LycT groups. No change in the expression of these genes was observed when the LycT group was compared to the control group.

Table 4 shows the protein expression of various genes related to proliferation during HCC in the different treatment groups. The expression of *HIF-1α* was significantly increased in the NDEA and LycT + NDEA groups when compared to the control and LycT groups. Significantly enhanced expression of *PCNA* and *Cyclin D1* was attributed to significantly higher absorbance at 405 nm in the NDEA group when compared to the control and LycT groups (Table 4). A significantly lower absorbance at 405 nm was observed for *PCNA* expression following LycT administration in NDEA-challenged mice when compared to the NDEA group. However, significantly enhanced expression of *PCNA* and *Cyclin D1* was observed in the LycT + NDEA group when compared to the control and LycT groups. Protein expression of *p21* was analyzed using ELISA in all treatment groups (Table 4). A significantly lower absorbance at 405 nm was found in the NDEA group when compared to the control and LycT groups. Significantly increased expression of *p21* was observed following LycT pre-treatment in NDEA-challenged mice when compared to the NDEA group. No significant change in the expression of *HIF-1α*, *PCNA*, *Cyclin D1*, and *p21* was observed between the LycT and control groups.

DISCUSSION

Previously, we observed that LycT yielded lycopene phyto-complex (LycT) which delayed and reduced the severity of NDEA-induced HCC as indicated by histopathology, tumor statistics and antioxidant defence system analysis[21]. The presence of a myriad number of compounds in the extract has been reported to enhance the medicinal properties of active components through synergistic effects[29,30]. The therapeutic activity of a medicinal plant is not due to a single component or a few components. However, one substance is so dependent on the presence of another substance that the plant or part of the plant when used in its entirety often yields better results than any single component if used in isolation. Lycopene extraction following basic solvent separation has been proved to be a better agent as there is substantial evidence to show that synergism further enhances its activity and efficacy[31]. Lycopene phyto-complex has also shown high efficacy in triggering apoptosis in addition to its anti-oxidative property[31]. However, the study would be incomplete if the effects of LycT on other hallmark pathways of HCC development were not demonstrated. One of the essential and necessary alterations for the development of almost all cancers is the induction of aerobic glycolysis (Warburg effect). Recently, scientists have linked sustained aerobic glycolysis to oncogenic mutations leading to abnormal cell proliferation and apoptosis[32]. Limited literature is available regarding the irreversible alterations in hepatic architecture during HCC development and their

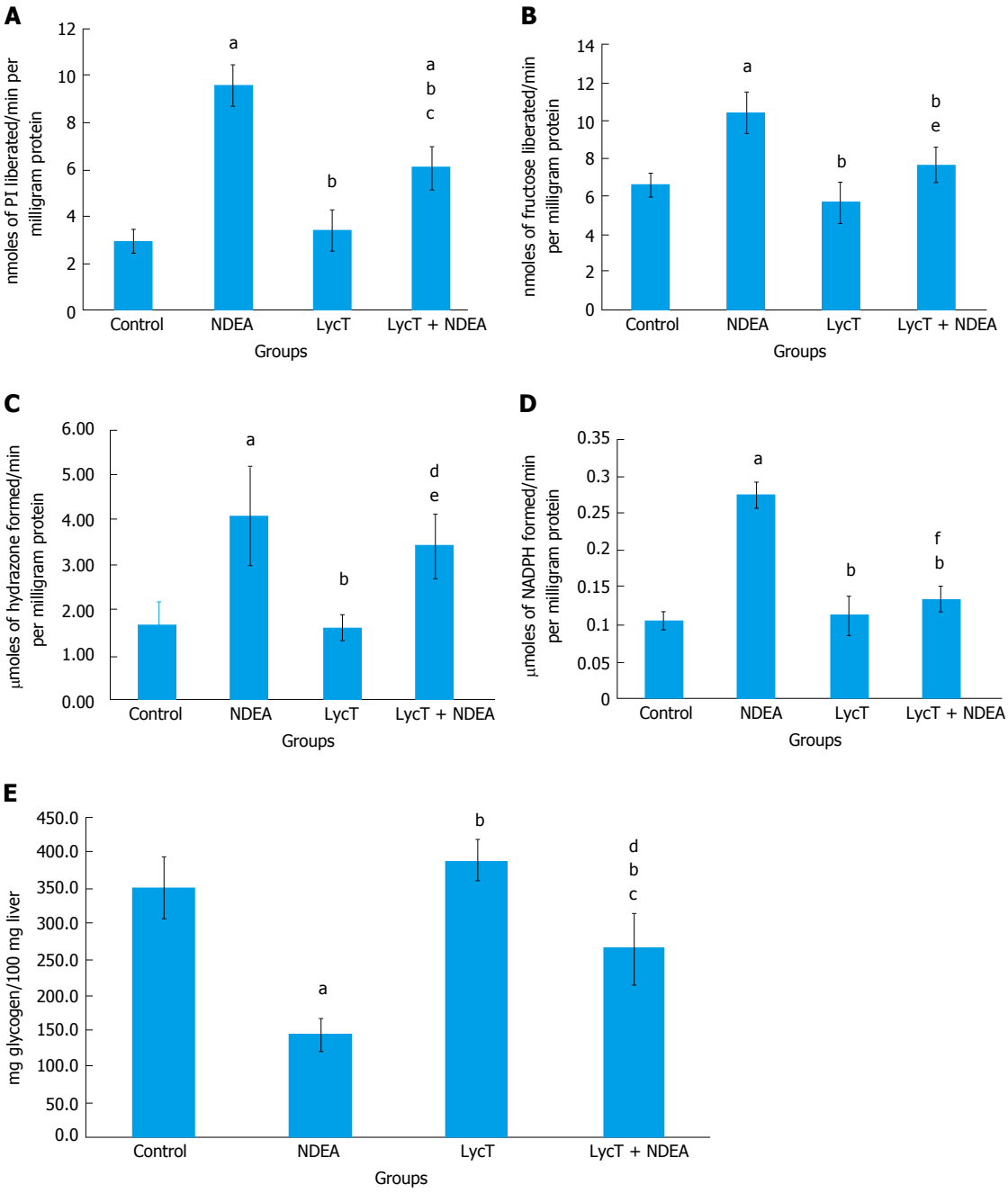
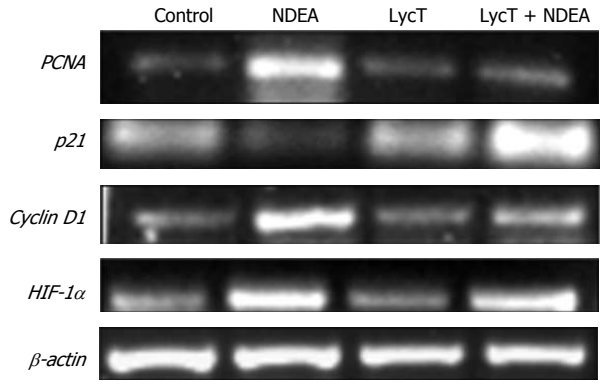


Figure 3 Effect of lycopene extracted from tomatoes and/or N-Nitrosodiethylamine. A: Hexokinase activity (nmoles of pseudo-inclusions liberated/min per milligram protein); B: Phosphoglucoisomerase (nmoles of fructose liberated/min per milligram protein); C: Aldolase (μmoles of hydrazone formed/min per milligram protein); D: G6PD (μmoles of NADPH formed/min per milligram protein); E: Glycogen (mg glucose/100 mg liver). $^{a}P \leqslant 0.05$, compared to the control group; $^{b,c,d}P \leqslant 0.001$, compared to the control group, NDEA group and LycT group, respectively; $^{e,f}P \leqslant 0.01$, compared to the control group and LycT group, respectively. LycT: Lycopene extracted from tomatoes; NDEA: N-Nitrosodiethylamine.

association with other dysregulated carcinogenic insults. With this in mind, the present study was designed to provide an insight into the alterations in ultrastructure, cell proliferation and aerobic glycolysis in NDEA-induced HCC and the effects of LycT on NDEA-induced HCC.

Several irreversible distortions using SEM and TEM were clearly observed and indicated the transformation of well-differentiated HCC to undifferentiated HCC in the NDEA group. Rapidly dividing tumor cells attaining a round contour during crowding of the cells has been reported in the literature[33]. Gross changes in nuclear morphology, epigenetic regulation, chromatin packing and overall nuclear architecture can be related to alterations in the molecular machinery[34]. However, LycT pre-treatment in NDEA-challenged mice resulted in reduced severity as depicted in micrographs. Increased lysosomal bodies and the presence of apoptotic bodies in hepatic tissue from the LycT + NDEA treated group revealed a high apoptotic rate and thus confirms the observations and strengthened our reported data. Aerobic glycolysis arises as a compensatory mechanism due to altered respiration in tumor cells to fulfil the ATP requirements during



Groups	Control	NDEA	LycT	LycT + NDEA
PCNA	0.46 ± 0.13	1.06 ± 0.26[a]	0.47 ± 0.13[b]	0.54 ± 0.12[b]
Cyclin D1	0.36 ± 0.01	0.72 ± 0.12[a]	0.35 ± 0.01[b]	0.50 ± 0.04[a,b,c]
p21	0.43 ± 0.03	0.17 ± 0.04[a]	0.46 ± 0.02[b]	0.77 ± 0.14[a,b,c]
HIF-1α	0.63 ± 0.02	1.06 ± 0.21[a]	0.59 ± 0.12[b]	0.93 ± 0.05[a,c]

Figure 4 Effect of lycopene extracted from tomatoes on mRNA expression of *PCNA*, *Cyclin D1*, *p21* and *HIF-1α* during N-Nitrosodiethylamine - induced hepatocarcinogenesis in mice. [a,b,c]$P \leqslant 0.001$, compared to the N-Nitrosodiethylamine group, LycT group and control group, respectively. LycT: Lycopene extracted from tomatoes; NDEA: N-Nitrosodiethylamine.

abnormal cell proliferation[35]. Similarly, enhanced hepatic hexokinase, PGI and aldolase activities in the NDEA group can be attributed to the high cell density observed. Such observations are in accordance with the available literature where enhanced glycolytic enzymes have been linked with chemically induced HCC[36]. Elevated levels of PGI have recently emerged as an excellent response to cancer and PGI level is used as a marker of metastatic growth in patients[37]. Moreover, significant reductions in the activities of glycolytic enzymes following LycT pre-treatment in NDEA-challenged mice were inversely related to HCC development. Histopathological and ultrastructural observations revealed well-differentiated HCC in the LycT + NDEA group, whereas poorly to undifferentiated HCC was observed in the NDEA group[21]. These structural observations could be correlated with the observed modulation of the glycolytic pathway. Various studies based on ^{18}F-FDG uptake on PET scans in different HCCs revealed that well differentiated HCC showed lower ^{18}F-FDG uptake in comparison with poorly differentiated HCC[38].

Ectopic expression of G6PD promotes the survival of tumor cells by maintaining both extracellular pH and redox potential[39]. Moreover, loss of p53 or mutated p53 has been linked with enhanced glucose consumption *via* increased activity of G6PD[40]. In the current study, LycT pre-treatment in NDEA-challenged mice resulted in significantly low expression of G6PD indicating the inhibitory role of lycopene in HCC by affecting metabolic pathways. Although there is limited research on the role of lycopene in regulating G6PD, some researchers have demonstrated the inhibitory effect of phytochemicals by regulating the activity of G6PD[41]. Decreased expression of Bcl-2 and enhanced p53 expression in the LycT + NDEA group may be responsible for reduced G6PD activity. In the current study, liver glycogen content in HCC was found to be decreased in the NDEA group. However, the reasons for the lack of glycogen accumulation were not fully explored. The transformation of liver cells to tumor cells causes a loss of glucose production *via* gluconeogenesis. According to the literature, overproduction of the molecule, microRNA-23a, is responsible for inhibiting gluconeogenesis[42]. Glycogen metabolism then acts as an alternate energy source, enabling growth of the cell under metabolic stress. A significant increase in the level of glycogen content was observed in the LycT + NDEA group when compared to the NDEA group. The current observations indicate that lycopene may interfere with glycogen conversion to glucose or might be due to less severe early HCC followed by lower ATP requirement. Such observations are in accordance with the previously reported effect of lycopene in CCl_4-challenged mice[43].

These observations in the NDEA group clearly point to a high rate of cell proliferation, which was further evident from increased *PCNA* and *Cyclin D1* expression. Enhanced proliferation attributed to increased expression of *PCNA* and *Cyclin D1* has been reported in the literature[44]. Decreased expression of *p21* in the NDEA group could be correlated with enhanced expression of *Cyclin D1* as *p21* is known to inhibit the activity of cyclin-CDKs complexes[45]. p21 is an important downstream mediator of p53 and its anti-proliferative property plays an important role in preventing tumor development. In our previous study it was observed that reduced p53 expression in the NDEA group was correlated with evasion of apoptosis[22]. Moreover, the LycT + NDEA group showed significantly enhanced expression of *p21* and reduced expression of *PCNA* when compared to the NDEA group indicating the anti-proliferative activity of LycT. Although it is difficult to comment on how lycopene or its metabolites inhibit HCC, the literature shows that treatment with lycopene or its metabolite increased *p21* expression and hence aided in preventing NDEA-induced cancer[46]. This observation is also supported by the current observation of increased cell density as shown by SEM and the hepatic glycolysis rate. Moreover, enhanced mRNA and protein expression of *HIF-1α* clearly indicated the existence of hypoxic conditions in NDEA-treated liver tissue. Various research groups have observed that the expression of *HIF-1* modulates apoptosis in HCC[11]. Reports have demonstrated that hypoxia enhances VEGF expression and decreases the ratio of Bax/Bcl-2, thus blocking apoptosis[47]. Pre-treatment with LycT in NDEA-challenged mice resulted in a significant reduction in the expression of *HIF-1α* at week 24 when compared to the NDEA group. Many reports have demonstrated similar observations where lycopene had an inhibitory response on HIF-1 in both *in vivo* and *in vitro* studies. Upadhyay *et al*[48] performed a comparative study of different antioxidants in order to assess their cancer preventive activity through the inhibition of HIF-1 activity. According to the results, HIF-1α operated in the presence of free radicals and antioxidants with maximum scavenging

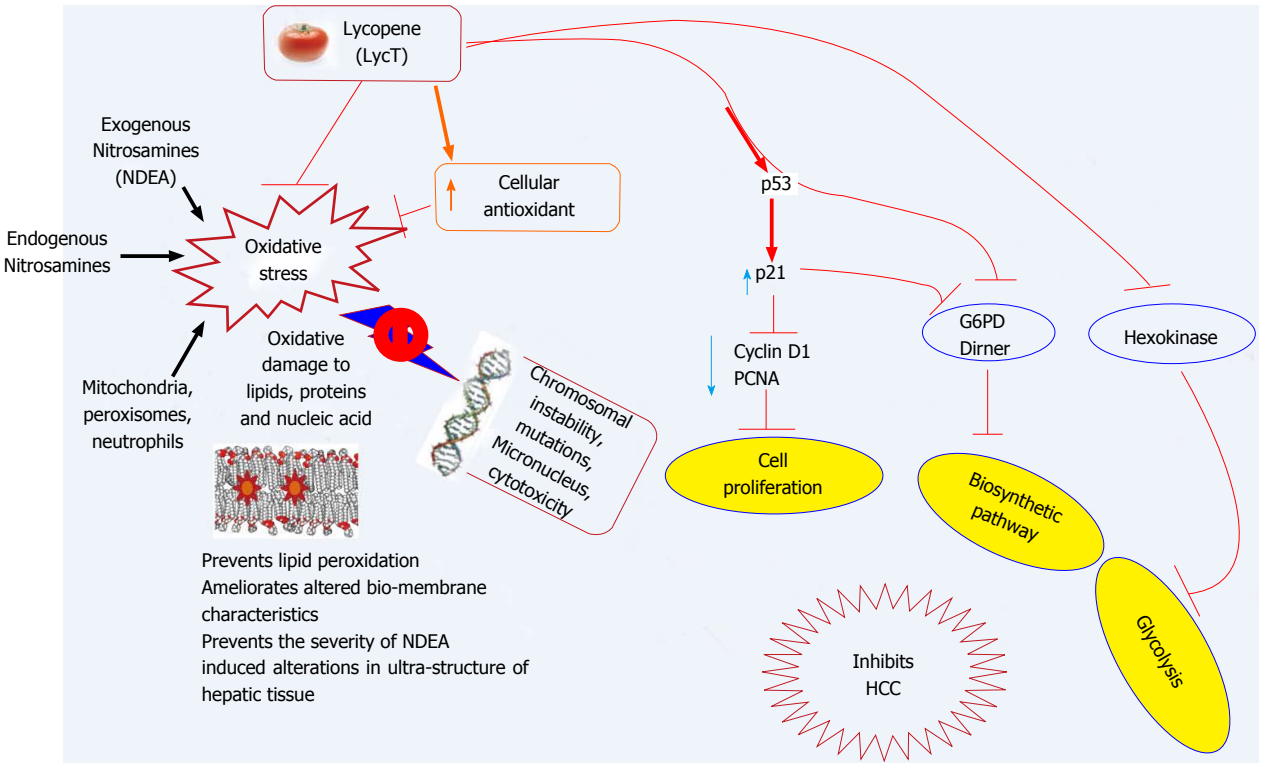


Figure 5 Overall mechanism of lycopene extracted from tomatoes mediated chemoprevention. LycT: Lycopene extracted from tomatoes; NDEA: N-Nitrosodiethylamine; HCC: Hepatocellular carcinoma; G6PD: Glucose-6-phosphate dehydrogenase.

efficiency for ROS cause inhibition of HIF-1α[49]. The literature also supports the consumption of tomatoes and lycopene mostly inhibited the expression of *HIF-1α* during prostate carcinogenesis[50]. Such reports strengthen our current observations, that the delay in HCC development may be attributed to the anti-proliferative effect of lycopene. In summary, our report demonstrates the potential of lycopene as a multi-targeted approach against chemically induced HCC.

Data from the present study and previously published studies show that LycT has beneficial effects against NDEA-induced HCC. Electron micrographs (SEM and TEM) of liver biopsies from the different treatment groups provided a picture of 3-D *in vivo* tissue modulations and thus served as an efficient and accurate tool for demonstrating carcinogenesis and the efficacy of chemopreventive agents along with histopathological observations. Moreover, aerobic glycolysis is also a potential target for determining the chemopreventive efficacy of lycopene and other phytochemicals in preventing carcinogenesis. As shown in the present study, LycT pre-treatment ameliorated disturbed metabolism, however, further studies are warranted to understand the in-depth pharmacokinetics and pharmacodynamics related to lycopene in experimental models of cancer. Finally, an attempt was made to represent diagrammatically the anti-carcinogenic effect of lycopene based on the current study and previous publications (Figure 5).

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COMMENTS

Background

Cancer is a complex disease and requires attention in multiple directions to prevent its development. Among the various carcinogenic insults, uncontrolled proliferation, dysregulated carbohydrate metabolism and hypoxia play very crucial roles in the development of hepatocellular carcinoma (HCC). Although there have been advances in therapeutic approaches a complete cure is still unavailable. Dietary multi-targeted agents have attracted the attention of cancer biologists as these agents may provide a solution to this complex problem by targeting multiple targets simultaneously. Lycopene, a polyunsaturated hydrocarbon imparting red colour to various fruits, is a nutritionally important carotenoid exhibiting beneficial health effects due to its antioxidant activity with minimal side effects. In addition to its antioxidant property, lycopene is known to modulate other non-oxidative pathways such as regulation of gap junction communication, the hormonal system, immune system and metabolic pathways of xenobiotics. Previously, we demonstrated that lycopene from tomatoes is a potent agent for inhibiting HCC development in terms of histopathological observations, tumor statistics, apoptosis, antioxidative capacity and toxicity. However, studies are warranted to determine the modulating effect of lycopene on dysregulated glucose metabolism and hypoxia, as these processes play critical roles in cancer. Thus, in the current study, the influence of lycopene extracted from tomatoes on various glycolytic and non-glycolytic enzymes, the expression of hypoxia inducible factor-1α (*HIF-1α*) and potent cell proliferation-associated genes while preventing N-Nitrosodiethylamine (NDEA)-induced HCC was investigated. Moreover, an attempt was made to demonstrate the impact of an imbalance between energy production and metabolic demands on the gross morphology and ultrastructure of hepatocytes in HCC.

Research frontiers

Important areas related to the current study include: (1) carcinogenesis: Incidence rate, statistics, prognosis, consequences, mortality, molecular and biochemical markers, altered cellular pathways and therapeutic limitations;

and (2) chemoprevention: Natural agents, multifaceted approach, lycopene a colored pigment and its antioxidative and anti-carcinogenic potential.

Innovations and breakthroughs

Despite significant research efforts, cancer is considered an incurable disease due to its high incidence rate, poor prognosis, high mortality rate, multifactorial causative agents, and side effects associated with chemotherapeutics. As natural agents are safer and have a multifaceted approach they are considered an alternative therapy. Lycopene is known to be a potent antioxidant and phytoagent with a protective effect against chronic diseases such as cancer. However, the detailed mechanism underlying its anti-carcinogenic effects is unclear. The basic requirement in the present investigation was to design a study that could demonstrate the action of lycopene at various stages to cover the maximum number of carcinogenic bioprocesses. The present study is part of this research design, in which hepatic tissue from different groups, *i.e.*, control, LycT, NDEA and LycT + NDEA groups was studied at different levels including structural markers, electro-physical markers, morphological markers, biochemical markers, and molecular markers that are known to be involved in the development of cancer. This type of study provides innovation and achievement for futuristic analyses of natural agents in disease models.

Applications

The outcomes of the current study provide deeper insight into the mechanistic targets of lycopene which shows ameliorating effects. Lycopene administration directly or indirectly stimulated various molecular targets such as p53, which have been correlated with decreased G6PD activity or biosynthetic pathways that play important roles during tumor proliferation. The protective effect of lycopene can be studied at the ultrastructural level by scanning and transmission electron microscopy (SEM and TEM). A detailed explanation regarding hepatic SEM and TEM during carcinogenesis is also information which is ambiguous in the literature.

Peer-review

This manuscript entitled "Lycopene modulates cellular proliferation, glycolysis and hepatic ultrastructure during hepatocellular carcinoma" investigated the effect of lycopene on ultra-structure, glycolytic enzymes, cell proliferation markers and hypoxia during NDEA induced hepatocarcinogenesis.

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ORIGINAL ARTICLE

Case Control Study

Polymorphisms of folate metabolism genes in patients with cirrhosis and hepatocellular carcinoma

Nathália Perpétua Peres, Ana Lívia Silva Galbiatti-Dias, Márcia Maria Urbanin Castanhole-Nunes, Renato Ferreira da Silva, Érika Cristina Pavarino, Eny Maria Goloni-Bertollo, Mariangela Torreglosa Ruiz-Cintra

Nathália Perpétua Peres, Ana Lívia Silva Galbiatti-Dias, Márcia Maria Urbanin Castanhole-Nunes, Érika Cristina Pavarino, Eny Maria Goloni-Bertollo, Mariangela Torreglosa Ruiz-Cintra, Genetics and Molecular Biology Research Unit, UPGEM, São José do Rio Preto 15090-000, Brazil

Nathália Perpétua Peres, Ana Lívia Silva Galbiatti-Dias, Márcia Maria Urbanin Castanhole-Nunes, Renato Ferreira da Silva, Érika Cristina Pavarino, Eny Maria Goloni-Bertollo, São José do Rio Preto Medical School, FAMERP and Medical School Fundation, FUNFARME, São José do Rio Preto 15090-000, Brazil

Renato Ferreira da Silva, Mariangela Torreglosa Ruiz-Cintra, Study Group of Liver Tumors, GETF, Hospital de Base, São José do Rio Preto 15090-000, Brazil

Mariangela Torreglosa Ruiz-Cintra, Federal University of the Triângulo Mineiro, UFTM, Uberaba 38064-200, Brazil

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Informed consent statement: Approved by that Ethics Research Committee.

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Data sharing statement: Patient data and full dataset are available with open accessfrom the corresponding author at analiviagalbiattidias@gmail.com. All Participants gave informed consent for data sharing. For more questions, please contact eny.goloni@famerp.br.

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Correspondence to: Eny Maria Goloni-Bertollo, MD, São José do Rio Preto Medical School, FAMERP and Medical School Fundation, FUNFARME, Av. Brigadeiro Faria Lima, 5416, São José do Rio Preto 15090-000, Brazil. eny.goloni@famerp.br
Telephone: +55-17-32015720

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Abstract

AIM

To evaluated the association of the risk factors and polymorphisms in *MTHFR C677T*, *MTHFR A1298C*, *MTR A2756G* and *MTRR A66G* genes.

METHODS

Patients with cirrhosis (n = 116), hepatocellular carcinoma (HCC) (n = 71) and controls (n = 356) were included. Polymerase chain reaction followed by enzymatic digestion and allelic discrimination technique real-time PCR techniques were used for analysis. MINITAB-14.0

and SNPstats were utilized for statistical analysis.

RESULTS

Showed that age ⩾ 46 years (OR = 10.31; 95%CI: 5.66-18.76; *P* < 0.001) and smoking (OR = 0.47; 95%CI: 0.28-0.78; *P* = 0.003) were associated with cirrhosis. Age ⩾ 46 years (OR = 16.36; 95%CI: 6.68-40.05; *P* < 0.001) and alcohol habit (OR = 2.01; 95%CI: 1.03-3.89; *P* = 0.039) were associated with HCC. *MTHFR A1298C* in codominant model (OR = 3.37; 95%CI: 1.52-7.50; *P* = 0.014), recessive model (OR = 3.04; 95%CI: 1.43-6.47; *P* = 0.0051) and additive model (OR = 1.71; 95%CI: 1.16-2.52; *P* = 0.0072) was associated with HCC, as well as *MTR A2756G* in the additive model (OR = 1.68; 95%CI: 1.01-2.77; *P* = 0.047), and *MTRR A66G* in the codominant model (OR = 3.26; 95%CI: 1.54-6.87; *P* < 0.001), dominant model (OR = 2.55; 95%CI: 1.24-5.25; *P* = 0.007) and overdominant model (OR = 3.05; 95%CI: 1.66-5.62; *P* < 0.001). *MTR A2756G* in the additive model (OR = 1.54; 95%CI: 1.02-2.33; *P* = 0.042) and smokers who presented at least one polymorphic allele for *MTRR A66G* (OR = 1.71; 95%CI: 0.77-3.82; *P* = 0.0051) showed increased risk for cirrhosis. There was no association between clinical parameters and polymorphisms.

CONCLUSION

Age ⩾ 46 years, alcohol habit and *MTR A2756G*, *MTHFR A1298C* and *MTRR A66G* polymorphisms are associated with an increased risk of HCC development; age ⩾ 46 years, tobacco habit and the *MTR A2756G* polymorphism are associated with cirrhosis.

Key words: Polymorphism; Folate metabolism; Liver cirrhosis; Hepatocellular carcinoma

Core tip: Our study is relevant because we can get better understanding on the mechanisms involved in the development of hepatocellular and Cirrhosis Carcinoma and folate metabolism. It is already known that polymorphisms cause DNA hypomethylation, which cause abnormal changes in gene expression inactivating suppressor genes tumor. In this study we have found some positive associations which was possible to understand the carcinogenesis of this tumor and offer new possibilities for diagnosis. Throughout these results it is possible to achieve better quality of life in early treatments.

Peres NP, Galbiatti-Dias ALS, Castanhole-Nunes MMU, da Silva RF, Pavarino ÉC, Goloni-Bertollo EM, Ruiz-Cintra MT. Polymorphisms of folate metabolism genes in patients with cirrhosis and hepatocellular carcinoma. *World J Hepatol* 2016; 8(29): 1234-1243 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i29/1234.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i29.1234

INTRODUCTION

Liver cancer is the second most common cause of death from cancer worldwide. Hepatocellular carcinoma (HCC) is considered the major form of primary liver cancer and is responsible for 70%-85% of all liver cancers[1]. Each year, more than half a million people are diagnosed with HCC. According to the most recent data, 782000 new cases per hundred thousand inhabitants have been diagnosed, with 745000 deaths resulting from this disease. HCC is the fifth most common cancer in men (554000 cases, 7.5% of all cases) and the ninth most common cancer in women (228000, 3.4% of all cases)[2].

The major risk factor for HCC development, present in 90% of HCC patients, is liver cirrhosis, which is characterized by diffuse fibrosis, progressive and irreversible, with the presence of nodules delimited by fibrous septa[1,3]. There are other risk factors such as hepatitis B and C virus infection, liver disease derived from alcohol consumption, exposure to toxins such as aflatoxins and smoking, non-alcoholic fatty liver, obesity and diabetes[4,5].

Cancer is a multifactorial disease that results from complex interactions between genetic and environmental factors[6]. Some studies have been conducted using genetic polymorphisms involved in folate metabolism in various types of cancers[7-11] because folate metabolism is essential for DNA synthesis and alterations in folate levels are associated with changes in DNA synthesis, methylation and repair, promoting genomic instability that contributes to the process of carcinogenesis[12].

Several enzymes, including methylenetetrahydrofolate reductase enzyme (MTHFR), methionine synthase (MTR) and methionine synthase reductase (MTRR), are involved in folate metabolism[13]. Methylenetetrahydrofolate reductase (MTHFR) is a key regulatory enzyme in folate metabolism, and MTHFR can catalyse 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is the predominant circulating form of folate. There are two common functional polymorphisms identified in the *MTHFR* gene, the *MTHFR C677T* polymorphism and *MTHFR A1298C* polymorphism[14].

Moreover, 5-methyl-tetrahydrofolate donates one methyl group for homocysteine remethylation to methionine. The remethylation of this reaction is catalysed by the enzyme methionine synthase (MTR), which requires vitamin B12 as a cofactor. The enzyme methionine synthase reductase (MTRR) is responsible for maintaining the active state of the MTR enzyme. Polymorphisms *MTR A2756G* and *MTRR A66G* may cause decreased activity of the enzyme, leading to increased plasma homocysteine and DNA hypomethylation, which causes changes in gene expression, inactivating tumour suppressor genes and activate oncogenesis[15-19].

Studies have confirmed that the genetic polymorphisms involved in folate metabolism may contribute to the development of HCC[16,19]. Therefore, the present

study was aimed to evaluate the association of risk factors and polymorphisms in the genes *MTHFR C677T*, *MTHFR A1298C*, *MTR A2756G* and *MTRR A66G* involved in folate metabolism with cirrhosis and HCC development in a case control study and to investigate the association of the polymorphisms with the clinical parameters of the disease in patients with cirrhosis and HCC.

MATERIALS AND METHODS

Ethical statement

Patients at the Liver, Intestine and Pancreas Transplant Unit of a university hospital in the northwest of the state of São Paulo, Brazil were included in the study after the diagnosis of liver cirrhosis and HCC, while the control group consisted of healthy individuals without cancer diagnosis. Individuals with a cancer family history were excluded from the control group. Informed consent was obtained from all subjects in this study, and the research protocol was approved by the Research Ethics Committee of FAMERP (CAAE: 20465713.1.0000.5415).

Patients

In this case-control study, 543 subjects (116 patients with liver cirrhosis, 71 patients with HCC and 356 healthy individuals) were included regardless of sex and age, from 2013 to 2015. Patients with cirrhosis were included because it is known as a well-established risk factor in 90% of patients with HCC[1,3].

The sample calculation was performed according the reports of Kwak *et al*[17] and Chang *et al*[19] which presented a similar sample calculation. Furthermore, no study has evaluated polymorphisms in folate metabolism in HCC and cirrhosis development in the Brazilian population.

The diagnosis of HCC was based on the criteria of the American Association for the Study of Liver Diseases published in 2012[20]. Liver biopsy was performed when the diagnosis was not possible by imaging methods, and the diagnosis of cirrhosis was made by clinical, laboratory, ultrasound and histopathological examinations when possible.

The variables analysed in this study were age, gender, exposure to risk factors (smoking and alcohol habit) and the presence of the *MTHFR A1298C, MTHFR C677T, MTR A2756G* and *MTRR A66G* polymorphisms. We considered smokers to be those who consumed at least 100 cigarettes during their lifetime and alcohol consumers to be those who drink more than 4 drinks weekly, corresponding 30 mL of liquor, 102 mL of wine, and 340 mL of beer[21].

Patients diagnosed with HCC were also classified according to the Barcelona Clinic Liver Cancer (BCLC) classification, which is a staging system that serves mainly for therapeutic guidance, in which the patient is ranked into five stages and includes other classifications. This classification uses variables related to tumour stage, the functional state of the liver, physical condition, and symptoms related to cancer. Patients with stage 0, very early HCC and with only a minor 2-cm tumour were nominated for liver resection. Patients with early HCC phase A with up to 3-cm nodules were eligible for curative therapies (resection, liver transplantation or percutaneous treatments). Patients in phase B with intermediate HCC and this multinodular underwent chemoembolization. Patients in advanced stage C presenting portal invasion and metastases received new agents such as sorafenib, which is a palliative treatment, and patients in stage D with end-stage disease received symptomatic treatment[22].

Methods

Genomic DNA was extracted from peripheral blood leukocytes of the cases and controls according to Miller *et al*[23] and was amplified by multiplex PCR-RFLP to identify the *MTHFR C677T* (rs1801133) and *MTHFR A1298C* (rs1801131), *MTR A2756G* (rs1805087) polymorphisms. The amplification product was subjected to digestion by the restriction enzymes Hinf Ⅰ, Mbo Ⅱ, and Hae Ⅲ, respectively. Electrophoresis was performed in 2.5% agarose gels at 110 volts for 100 min. Allelic discrimination *via* the Real-Time PCR - SNP Genotyping Assay (Applied Biosystems) was used to identify the *MTRR A66G* (rs1801394) polymorphism, using primers and probes specific for each allele available by the manufacturer (MTRR A66G: C_3068176_10)[7].

Genotyping confirmation was accomplished in 10% random samples of each group, and 100% concordance was observed.

Statistical analysis

Hardy-Weinberg equilibrium (HWE) was performed using χ^2 test. The multiple regression logistic test by the Minitab program - Version 14.0 was used to determine the effects of variables. The model evaluated the following variables: Age (reference: < 46 years; median), smoking habits (reference: No smokers), alcohol habit (reference: Non-consumers) and gender (reference: Female). The polymorphisms were used to adjust the analysis.

The multiple logistic regression model adjusted for age, gender, smoking and alcohol habits was also used to assess the association between polymorphisms and the development of cirrhosis and HCC using the SNPStats program. The effect of the polymorphisms was evaluated in the following models: (1) codominant (heterozygous *vs* homozygous wild type and polymorphic homozygous *vs* homozygous wild type); (2) dominant (heterozygous more polymorphic homozygous *vs* homozygous wild type); (3) recessive (polymorphic homozygous *vs* homozygous wild type more heterozygous); (4) overdominant (wild homozygous *vs* heterozygous more polymorphic homozygote); and (5) additive (weight polymorphic homozygote *vs* heterozygote 2 more homozygous wild-type).

The SNPStats program was also used to assess the

Table 1 Relationship between risk factors and hepatocellular carcinoma and liver cirrhosis development

Variables	Controls *n* (%)	Cirrhosis *n* (%)	[1]OR (95%CI)	*P*-value	HCC *n* (%)	OR (95%CI)	[2]*P*
Age							
< 46 anos	238 (67)	22 (19)	10.31 (5.66-18.76)	$P < 0.001$	8 (11)	16.36 (6.68-40.05)	$P < 0.001$
⩾ 46 anos	118 (33)	94 (81)			63 (89)		
Genre							
Female	95 (26.7)	30 (25.9)	0.96 (0.54-1.72)	$P = 0.893$	19 (26.8)	0.59 (0.29-1.22)	$P = 0.154$
Male	261 (73.3)	86 (74.1)			52 (73.2)		
Alcoholic habit							
Not	191 (54)	54 (46.6))	1.55 (0.91-2.63)	$P = 0.106$	27 (38)	2.01 (1.03-3.89)	$P = 0.039$
Yes	165 (46)	62 (53.4)			44 (62)		
Smoking habit							
Nonsmokers	199 (56)	72 (62)	0.47 (0.28-0.78)	$P = 0.003$	31 (43.7)	0.9 (0.48-1.67)	$P = 0.734$
Smokers	157 (44)	44 (38)			40 (56.3)		

[1]Odds ratio (OR) adjusted for age, genre, alcohol consumption, smoking habits and polymorphisms; [2]*P* values significant at $P \leqslant 0.05$. HCC: Hepatocellular carcinoma.

potential interaction between the polymorphisms with variables associated with cirrhosis and HCC development (tobacco and alcohol habits) through multiple logistic regression.

The *MTHFR* haplotypes were inferred using the Haploview 4.2 statistical program, which creates population frequency estimates of the haplotypes.

The association between the clinical parameters and polymorphisms with HCC development were also analysed by multiple logistic regression. The patients were subjected to classification and BCLC staging divided into five stages (0, A, B, C and D). The variables alpha fetoprotein dose values, hepatitis B and C, diabetes mellitus and death were utilized in the adjustment of the analysis. The models included BCLC classification (reference: 0, A), alpha fetoprotein (reference: < 500 ng/mL), hepatitis B (reference: No), hepatitis C virus (reference: No), diabetes (reference: Absence), death (reference: No) and the studied polymorphisms (reference: Wild-type genotype).

The Kaplan-Meier method was applied to evaluate the survival rate by considering the period between the disease diagnosis and death to be the end point.

The results were presented as ORs and 95%CIs. The level of significance was set at 5% ($P = 0.05$).

RESULTS

The results for HWE were similar to those expected in both the case and control groups, respectively, for the *MTHFR C677T* ($\chi^2 = 0.8940$, $P = 0.3444$ and $\chi^2 = 3.1218$, $P = 0.0772$), *MTR A2756G* ($\chi^2 = 1.1554$, $P = 0.2824$ and $\chi^2 = 1.1929$, $P = 0.2748$) and *MTRR A66G* polymorphisms ($\chi^2 = 3$, 2227, $P = 0.0726$ and $\chi^2 = 0.0530$, $P = 0.8018$). However, the *MTHFR A1298C* polymorphism showed no equilibrium ($\chi^2 = 8.0244$, $P = 0.0046$ and $\chi^2 = 8.6427$, $P = 0.0033$) for patients with HCC and/or cirrhosis and controls.

Table 1 shows the results for multiple logistic regression analysis between patients with liver cirrhosis and control subjects to determine the effects of variables. Age ⩾ 46 years (OR = 10.31; 95%CI: 5.66-18.76; $P < 0.001$) and smoking habit were associated with the disease (OR = 0.47; 95%CI: 0.28-0.78; $P = 0.003$), and the analysis of patients with HCC and control subjects showed that age ⩾ 46 years (OR = 16.36; 95%CI: 6.68-40.05; $P < 0.001$) and alcohol habit (OR = 2.01; 95%CI: 1.03-3.89; $P = 0.039$) were associated with the disease.

Table 2 shows the association of the *MTHFR C677T, MTHFR A1298C, MTR A2756G* and *MTRR A66G* polymorphisms with HCC, adjusted for gender, age, smoking and alcohol habit according to the heritage models. The *MTHFR A1298C* polymorphism in the codominant model (OR = 3.37; 95%CI: 1.52-7.50; $P = 0.014$), recessive model (OR = 3.04; 95%CI: 1.43-6.47; $P = 0.0051$) and additive model (OR = 1.71; 95%CI: 1.16-2.52; $P = 0.0072$), the *MTR A2756G* polymorphism in the additive model (OR = 1.68; 95%CI: 1.01-2.77; $P = 0.047$), the *MTRR A66G* polymorphism in the codominant model (OR = 3.26; 95%CI: 1.54-6.87; $P < 0.001$), dominant model (OR = 2.55; 95%CI: 1.24-5.25; $P = 0.007$) and overdominant model (OR = 3.05; 95%CI: 1.66-5.62; $P < 0.001$) were associated with an increased risk of HCC development.

Table 3 shows the association of the *MTHFR C677T, MTHFR A1298C, MTR A2756G* and *MTRR A66G* polymorphisms with liver cirrhosis, adjusted for gender, age, smoking and alcohol habit according to the heritage models. The *MTR A2756G* polymorphism was associated with an increased risk of liver cirrhosis in the additive model (OR = 1.54; 95%CI: 1.02-2.33; $P = 0.042$).

Regarding the potential interaction among the polymorphisms with variables associated with the diseases, there was no interaction among the *MTHFR C677T*, *MTHFR A1298C*, *MTR A2756G* and *MTRR A66G* polymorphisms and smoking habit or alcohol habit regarding the risk of HCC (Table 4). However, smokers who presented with the heterozygous genotype (AG) or polymorphic homozygote genotype (GG) for the MTRR gene (OR = 1.71; 95%CI: 0.77-3.82; $P = 0.0051$) was associated with liver cirrhosis (Table 5).

The haplotype analysis showed a higher frequency

Table 2 Association of *MTHFR C677T, MTHFR A1298C, MTR A2756G* and *MTRR A66G* polymorphisms with hepatocellular carcinoma, adjusted for gender, age, smoking and alcohol consumption

Model	Genotipe	Control *n* (%)	Case *n* (%)	[1]OR (95%CI)	[2]*P*-value	Genotipe	Control *n* (%)	Case *n* (%)	[1]OR (95%CI)	[2]*P*-value
	MTHFR C677T					*MTHFR A1298C*				
Codominant	C/C	149 (41.9)	28 (39.4)	1		A/A	205 (57.6)	32 (45.1)	1	
	C/T	174 (48.9)	36 (50.7)	0.93 (0.51-1.68)	0.91	A/C	116 (32.6)	24 (33.8)	1.29 (0.69-2.42)	0.014
	T/T	33 (9.3)	7 (9.9)	1.13 (0.41-3.09)		C/C	35 (9.8)	15 (21.1)	3.37 (1.52-7.50)	
Dominant	C/C	149 (41.9)	28 (39.4)	1		A/A	205 (57.6)	32 (45.1)	1	
	C/T-T/T	207 (58.1)	43 (60.6)	0.96 (0.54-1.70)	0.88	A/C-C/C	151 (42.4)	39 (54.9)	1.71 (0.98-2.99)	0.06
Recessive	C/C-C/T	323 (90.7)	64 (90.1)	1		A/A-A/C	321 (90.2)	56 (78.9)	1	
	T/T	33 (9.3)	7 (9.9)	1.18 (0.46-3.05)	0.73	C/C	35 (9.8)	15 (21.1)	3.04 (1.43-6.47)	0.0051
Overdominant	C/C-T/T	182 (51.1)	35 (49.3)	1		A/A-C/C	240 (67.4)	47 (66.2)	1	
	C/T	174 (48.9)	36 (50.7)	0.91 (0.52-1.59)	0.73	A/C	116 (32.6)	24 (33.8)	0.99 (0.55-1.78)	0.98
Aditive	---	---	---	1.01 (0.64-1.58)	0.97	---	---	---	1.71 (1.16-2.52)	0.007
	MTR A2756G					*MTRR A66G*				
Codominant	A/A	263 (73.9)	46 (64.8)	1		A/A	105 (29.5)	12 (16.9)	1	
	A/G	83 (23.3)	21 (29.6)	1.58 (0.84-2.98)	0.13	A/G	179 (50.3)	50 (70.4)	3.26 (1.54-6.87)	< 0.001
	G/G	10 (2.8)	4 (5.6)	3.29 (0.81-13.30)		G/G	72 (20.2)	9 (12.7)	1.16 (0.44-3.11)	
Dominant	A/A	263 (73.9)	46 (64.8)	1		A/A	105 (29.5)	12 (16.9)	1	
	A/G-G/G	93 (26.1)	25 (35.2)	1.73 (0.95-3.15)	0.078	A/G-G/G	251 (70.5)	59 (83.1)	2.55 (1.24-5.25)	0.0072
Recessive	A/A-A/G	346 (97.2)	67 (94.4)	1		A/A-A/G	284 (79.8)	62 (87.3)	1	
	G/G	10 (2.8)	4 (5.6)	2.91 (0.73-11.59)	0.15	G/G	72 (20.2)	9 (12.7)	0.51 (0.23-1.12)	0.077
Overdominant	A/A-G/G	273 (76.7)	50 (70.4)	1		A/A-G/G	177 (49.7)	21 (29.6)	1	
	A/G	83 (23.3)	21 (29.6)	1.49 (0.80-2.79)	0.22	A/G	179 (50.3)	50 (70.4)	3.05 (1.66-5.62)	< 0.001
Aditive	---	---	---	1.68 (1.01-2.77)	0.047	---	---	---	1.16 (0.77-1.73)	0.48

[1]Odds ratio (OR) adjusted for age, gender and alcohol consumption and smoking habits; [2]*P* values significant at $P \leqslant 0.05$.

Table 3 Association of *MTHFR C677T, MTHFR A1298C, MTR A2756G* and *MTRR A66G* polymorphisms with Liver Cirrhosis, adjusted for gender, age, smoking and alcohol consumption

Model	Genotipe	Control *n* (%)	Case *n* (%)	[1]OR (95%CI)	[2]*P*-value	Genotipe	Control *n* (%)	Case *n* (%)	[1]OR (95%CI)	[2]*P*-value
	MTHFR C677T					*MTHFR A1298C*				
Codominant	C/C	149 (41.9)	48 (41.4)	1		A/A	205 (57.6)	57 (49.1)	1	
	C/T	174 (48.9)	55 (47.4)	0.90 (0.56-1.45)	0.56	A/C	116 (32.6)	43 (37.1)	1.27 (0.78-2.07)	0.21
	T/T	33 (9.3)	13 (11.2)	1.37 (0.64-2.95)		C/C	35 (9.8)	16 (13.8)	1.85 (0.92-3.71)	
Dominant	C/C	149 (41.9)	48 (41.4)	1		A/A	205 (57.6)	57 (49.1)	1	
	C/T-T/T	207 (58.1)	68 (58.6)	0.97 (0.62-1.52)	0.89	A/C-C/C	151 (42.4)	59 (50.9)	1.40 (0.90-2.18)	0.14
Recessive	C/C-C/T	323 (90.7)	103 (88.8)	1		A/A-A/C	321 (90.2)	100 (86.2)	1	
	T/T	33 (9.3)	13 (11.2)	1.45 (0.71-2.99)	0.32	C/C	35 (9.8)	16 (13.8)	1.68 (0.86-3.29)	0.14
Overdominant	C/C-T/T	182 (51.1)	61 (52.6)	1		A/A-C/C	240 (67.4)	73 (62.9)	1	
	C/T	174 (48.9)	55 (47.4)	0.85 (0.54-1.33)	0.47	A/C	116 (32.6)	43 (37.1)	1.14 (0.72-1.82)	0.58
Aditive	---	---	---	1.07 (0.75-1.51)	0.72	---	---	---	1.33 (0.97-1.83)	0.079
	MTR A2756G					*MTRR A66G*				
Codominant	A/A	263 (73.9)	79 (68.1)	1		A/A	105 (29.5)	37 (31.9)	1	
	A/G	83 (23.3)	32 (27.6)	1.52 (0.91-2.53)	0.13	A/G	179 (50.3)	55 (47.4)	0.94 (0.56-1.56)	0.95
	G/G	10 (2.8)	05 (4.3)	2.47 (0.75-8.12)		G/G	72 (20.2)	24 (20.7)	0.91 (0.49-1.71)	
Dominant	A/A	263 (73.9)	79 (68.1)	1		A/A	105 (29.5)	37 (31.9)	1	
	A/G-G/G	93 (26.1)	37 (31.9)	1.60 (0.98-2.61)	0.06	A/G-G/G	251 (70.5)	79 (68.1)	0.93 (0.58-1.50)	0.77
Recessive	A/A-A/G	346 (97.2)	111 (95.7)	1		A/A-A/G	284 (79.8)	92 (79.3)	1	
	G/G	10 (2.8)	5 (4.3)	2.19 (0.68-7.09)	0.21	G/G	72 (20.2)	24 (20.7)	0.95 (0.55-1.64)	0.86
Overdominant	A/A-G/G	273 (76.7)	84 (72.4)	1		A/A-G/G	177 (49.7)	61 (52.6)	1	
	A/G	83 (23.3)	32 (27.6)	1.46 (0.88-2.41)	0.15	A/G	179 (50.3)	55 (47.4)	0.97 (0.62-1.52)	0.9
Aditive	---	---	---	1.54 (1.02-2.33)	0.042	---	---	---	0.95 (0.70-1.30)	0.77

[1]Odds ratio (OR) adjusted for age, gender and alcohol consumption and smoking habits; [2]*P* values significant at $P \leqslant 0.05$.

(40.6%) of the AC haplotype observed in both groups (Case group: 0.403, Control group: 0.407; $\chi^2 = 0.01$, $P = 0.9194$). The haplotype frequencies of AT (Case group: 0.283, Control group: 0.312; $\chi^2 = 0.843$, $P = 0.3584$), haplotype frequencies of CC (Case group: 0.269, Control group: 0.247; $\chi^2 = 0.573$, $P = 0.4491$), and haplotype frequencies of CT (Case group: 0.045, Control group: 0.035; $\chi^2 = 0.569$, $P = 0.4505$) did not show significant

Table 4 Interaction between *MTHFR C677T*, *MTHFR A1298C*, *MTR A2756G* and *MTRR A66G* polymorphisms and smoking habits or alcohol drinking on the risk of hepatocellular carcinoma

	Smoking habits							Alcoholic habit						
	No smoker			Smoker			^2P interaction	Non-alcoholic			Alcoholic			^2P interaction
	Case	Control	^{1}OR (95%CI)	Case	Control	^{1}OR (95%CI)		Case	Control	^{1}OR (95%CI)	Case	Control	^{1}OR (95%CI)	
MTRA2756G														
A/A	22	142	1.00	24	121	1.00	0.81	17	137	1.00	29	126	1.00	0.43
A/G-G/G	10	57	1.59 (0.65-3.87)	15	36	1.85 (0.81-4.21)		11	53	2.29 (0.91-5.72)	14	40	1.40 (0.63-3.12)	
MTRRA66G														
A/A	4	55	1.00	8	50	1.00	0.7	3	53	1.00	9	52	1.00	0.34
A/G-G/G	28	144	3.04 (0.95-9.71)	31	107	2.27 (0.90-5.72)		25	137	4.16 (1.11-15.55)	34	114	1.98 (0.83-4.75)	
MTHFRC677T														
C/C	15	85	1.00	13	64	1.00	0.55	14	85	1.00	14	64	1.00	0.75
C/T-T/T	17	114	0.80 (0.35-1.81)	26	93	1.13 (0.51-2.52)		14	105	0.86 (0.36-2.06)	29	102	1.04 (0.48-2.22)	
MTHFRA1298C														
A/A	13	119	1.00	19	85	1.00	0.61	11	113	1.00	21	91	1.00	0.56
A/C-C/C	19	80	2.00 (0.88-4.56)	20	72	1.49 (0.69-3.20)		17	77	2.10 (0.86-5.10)	22	75	1.49 (0.72-3.07)	

^{1}Odds ratio (OR) adjusted for age, gender and alcohol consumption and smoking habits; ^2P values significant at $P \leqslant 0.05$.

Table 5 Interaction between *MTHFR C677T*, *MTHFR A1298C*, *MTR A2756G* and *MTRR A66G* polymorphisms and smoking habits or alcohol drinking on the risk of liver cirrhosis

	Smoking habits							Alcoholic habit						
	No smoker			Smoker			^2P interaction	Non-alcoholic			Alcoholic			^2P interaction
	Case	Control	^{1}OR (95%CI)	Case	Control	OR$^+$ (95%CI)		Case	Control	^{1}OR (95%CI)	Case	Control	^{1}OR (95%CI)	
MTRA2756G														
A/A	50	142	1.00	29	121	1.00	0.39	36	137	1.00	43	126	1.00	0.73
A/G-G/G	21	57	1.35 (0.72-2.53)	16	36	2.07 (0.97-4.41)		17	53	1.46 (0.72-2.97)	20	40	1.74 (0.89-3.38)	
MTRRA66G														
A/A	26	55	1.00	11	50	1.00	0.051	14	53	1.00	23	52	1.00	0.42
A/G-G/G	45	144	0.63 (0.35-1.17)	34	107	1.71 (0.77-3.82)		39	137	1.17 (0.56-2.45)	40	114	0.78 (0.42-1.48)	
MTHFRC677T														
C/C	31	85	1.00	17	64	1.00	0.96	24	85	1.00	24	64	1.00	0.76
C/T-T/T	40	114	0.96 (0.54-1.71)	28	93	0.98 (0.48-2.02)		29	105	1.05 (0.54-2.02)	39	102	0.91 (0.49-1.69)	
MTHFRA1298C														
A/A	33	120	1.00	24	85	1.00	0.32	23	113	1.00	34	92	1.00	0.56
A/C-C/C	38	79	1.68 (0.95-3.00)	21	72	1.06 (0.53-2.13)		30	77	1.83 (0.95-3.54)	29	74	1.11 (0.61-2.03)	

^{1}Odds ratio (OR) adjusted for age, gender and alcohol consumption and smoking habits; ^2P values significant at $P \leqslant 0.05$.

results.

There was no association in the multiple logistic regression analysis of the analysed clinical parameters and polymorphisms in patients with HCC stratified into tumours in stages 0 and A, and tumours in stages B, C and D, according to the BCLC criteria (Table 6).

The Kaplan-Meier survival curves for genotype showed no association of polymorphisms and overall survival with HCC development. No polymorphism was associated (*MTHFR C677T*, $P = 0.5483$; *MTHFR A1298C*, $P = 0.3861$; *MTR A2756G*, $P = 0.6765$; *MTRR A66G*, $P = 0.3840$) with overall survival.

DISCUSSION

The results showed that age $\geqslant$ 46 years and alcohol habit were associated with an increased risk of HCC development, similar to the results of Fassio *et al*[24], Varela *et al*[25], Munaka *et al*[26], Carrilho *et al*[3], Donato *et al*[27], Hamed *et al*[28] and Mittal *et al*[1].

Brazilian publications have reported that the mean age of the HCC patients is 54.6 years[3]; in Latin

Table 6 Regression analysis of data from multiple logistic analyzed clinical parameters and polymorphisms in patients with hepatocellular carcinoma tumors divided into stages 0 and tumore in stages A and B, C and D according barcelona clinic liver cancer criteria

Variables	Stage 0 e A Pacientes *n* (%)	Estage B, C e D Pacientes *n* (%)	OR (95%CI)[1]	*P*
Alpha fetoprotein				
> 500 ng/mL	22 (84.6)	21 (46.7)	Reference	Reference
< 500 ng/mL	4 (15.4)	24 (53.3)	2.66 (0.55-12.72)	0.22
Hepatitis B virus				
Absence	25 (96.2)	38 (84.4)	Reference	Reference
Presence	1 (3.85)	7 (15.6)	4.06 (0.34-48.29)	0.27
Hepatitis C virus				
Absence	12 (46.2)	22 (48.9)	Reference	Reference
Presence	14 (53.8)	23 (41.1)	1.43 (0.35-5.78)	0.61
Steatohepatitis				
Absence	26 (100)	42 (93.3)	Reference	Reference
Presence	00 (00)	3 (6.7)	[3]	0.99
Diabetes				
Absence	18 (69.2)	32 (71.1)	Reference	Reference
Presence	8 (30.8)	13 (28.9)	0.25 (0.03-1.63)	0.15
Death				
No	24 (92.3)	17 (37.8)	Reference	Reference
Yes	2 (7.7)	28 (62.2)	25.3 (3.67-174.38)	0.001[2]
MTHFR A1298C				
AA	10 (38.5)	22 (48.9)	Reference	Reference
AC/CC	16 (61.5)	23 (51.1)	0.93 (0.22-3.86)	0.92
MTHFR C677T				
CC	11 (42.3)	17 (37.8)	Reference	Reference
CT/TT	15 (57.7)	28 (62.2)	1.65 (0.34-7.97)	0.53
MTR A2756G				
AA	18 (69.2)	28 (62.2)	Reference	Reference
AG/GG	8 (30.8)	17 (37.8)	0.78 (0.18-3.43)	0.75
MTRRA66G				
AA	4 (15.4)	8 (17.8)	Reference	Reference
AG/GG	22 (84.6)	37 (82.2)	1.09 (0.16-7.30)	0.93

[1]OR: Odds ratio, CI: Confidence interval; [2]Statistically significant at $P \leqslant 0.05$; [3]Could not calculate due to numerical proximity.

America, the average age in one published study was 64 years[24]; in another multicentre study in Spain, the reported average age was 65.6 years[25], and Mittal *et al*[1] concluded that a more recent increase in the incidence of HCC in the United States population was seen in Hispanics and blacks between the ages of 45 and 65 years, results that are similar to ours.

Regarding the result that alcohol consumption was also significant and more frequent in patients with HCC, a prospective case-control study from Japan has observed that heavy alcohol drinkers had a five-fold increase in the risk of HCC compared with non-drinkers[26]. Donato *et al*[27] 2002 in Italy, with a sample size of 464 cases and 824 controls individuals, found a positive relationship between alcohol consumption and HCC. The latter findings were confirmed in review of Hamed *et al*[28].

Evidence of a positive association between heavy alcohol drinking and liver cancer is derived mainly from case-control studies. The increased risk of those drinking 6 or more drinks per day compared with non-drinkers was 22%. Alcohol was the only cause present in 14% of cases in the 2010 Brazilian study by Carrilho *et al*[3]. Thus, the significance indexes are increased because drinking alcohol causes poor absorption of vitamin B complex, changing the folate metabolism and causing oxidative damage and breaks in the DNA strands[29]. In our study we found the association between alcohol and HCC development, this may be due the fact described above.

Regarding cirrhosis, we found that age $\geqslant$ 46 years and smoking habit was associated with risk of cirrhosis. As previously mentioned, 85%-90% of primary liver cirrhosis causes cancer, and multiple nonviral factors that are concerned with the development of liver cancer include iron overload syndromes, alcohol use, tobacco, oral contraceptives, aflatoxin, and pesticide exposure, which is prevalent in the developing world[28]. Regarding the tobacco habit, the data showed that 38% of individuals with cirrhosis had a tobacco habit. In addition to the liver, which is the target of chemical compounds in tobacco that can progress to cirrhosis, it was also observed in the literature that the development of diseases related to the progression to cirrhosis occurs more frequently in older individuals[30].

In addition to the association that we found between tobacco habit and cirrhosis development, there can be a relationship of tobacco with the dysfunction of genes as

well as enzymes involved in the detoxification of nicotine, consequences that generate various types of liver-related diseases such as fibrosis, alcoholic hepatitis, cirrhosis and HCC[31]. Although alcohol habit is a well-established risk factor for cirrhosis development[32], our study did not find this association. However, 53.4% of cirrhosis patients in the present study were alcohol consumers.

In relation to the genetic characteristics, the present study was the first to be performed in a Brazilian population with HCC and cirrhosis and revealed that the *MTR A2756G, MTHFR A1298C* and *MTRR A66G* polymorphisms were associated with an increased risk of HCC development, results similar to the studies of Kwak *et al*[17] and Yu *et al*[33].

Chang *et al*[19], with a sample of 204 patients with liver cancer and 415 controls found an association between *MTR A2756G* and increased risk for the disease, as well the meta-analysis performed by Yu *et al*[33] that reported a significantly higher association between the genotype and 2756GG cancer risk in Asian populations.

There are studies involving other cancers that have found a positive association with at least one polymorphic allele 2756G and an increased risk of the development of disease. For example, the Hosseini *et al*[8] that evaluated 592 individuals in Iran found an association between in MTR GG genotype and breast cancer; Galbiatti *et al*[7] also concluded that *MTR A2765G* polymorphism is involved in the risk of head and neck cancer; de Lima *et al*[9] suggested an association between the *MTR A2756G* polymorphism and retinoblastoma susceptibility in a northeast population of Brazil, Ouerhani *et al*[10] found that *MTR A2756G* affecting bladder cancer risk.

Regarding the *MTRR A66G* polymorphism, Kwak *et al*[17] studied 96 patients and 201 controls and observed an association between the polymorphism and an increased risk of HCC, a finding that has also been found in other types of cancer; the Wu *et al*[11] study demonstrated a positive relationship with the *MTRR A66G* polymorphism and breast cancer, and a meta-analysis performed by Zhou *et al*[34] also found an association between this polymorphism and colorectal cancer, which is in agreement with to our study. However, the study of Zhang *et al*[35] did not find an association of this polymorphism with HCC development.

We also found an association between the *MTHFR A1298C* polymorphism and an increased risk of HCC development. Two meta-analyses reported an association of this polymorphism with a decreased risk of HCC, demonstrating a protective effect[36,37]. However, Liang *et al*[38] meta-analysis of a total of seven studies showed that the homozygote genotype CC of the MTHFR rs1801131 polymorphism was significantly associated with a decreased risk of liver cancer (for CC *vs* AA: OR = 0.65, 95%CI: 0.47-0.89, $P = 0.007$; for CC *vs* AA + AC: OR = 0.65, 95%CI: 0.48-0.89, $P = 0.006$), similar our study.

Our results for cirrhosis and polymorphisms showed an association between *MTR A2756G* and an increased risk of the disease. There are no studies in the literature that have evaluated the association between the *MTR A2756G* polymorphism and cirrhosis development. The present study is the first to investigate the *MTRA A2756G* polymorphism and cirrhosis development, and the association that was found can be related to alteration of the MTR enzyme that occurs due to the presence of the *MTR A2756G* polymorphism. The alteration of the MTR enzyme causes elevation in the homocysteine levels and DNA hypomethylation, leading to chromosomal instability, mutations and the overexpression of proto-oncogenes that can be associated with the development of several types of diseases, including cirrhosis. However, more studies in different populations of individuals with cirrhosis are needed[39].

Regarding the potential interaction among the polymorphisms with variables associated with the diseases, we found that smoking in those with the heterozygous genotype (AG) or polymorphic homozygote genotype (GG) for *MTRR* gene was associated with liver cirrhosis. There are no studies that have investigated this interaction, however, tobacco habit can be related to cirrhosis because the chemical compounds can modify the liver and lead to cirrhosis[39] independently of the *MTRR A66G* polymorphism.

Regarding BCLC classification, we did not find an association with the polymorphisms evaluated. Our data showed that 7% of patients in stage 0, 29.6% in stage A, 22.5% in stage B, 31% in stage C and 9.8% in stage D. The study of Varela *et al*[25] reported that 49.8% of 705 cases were in the initial stage (A), 19.8% in the intermediate stage (B), 18.8% in the advanced stage (C) and 11.6% in the terminal phase (D). Additionally, the study of Raphe *et al*[40] reported that 32.7% were in stage A, 22% in stage B, 30.4% in stage C, 14% in stage D. Current published data show that patients who are in stage A are asymptomatic and have preserved liver function have a 5-year survival of 50%-75%. Patients who are in stage B have a median survival of 20 mo; those who are already in the C and D stages have severe liver dysfunction and extrahepatic metastases reach an 11-mo survival, and only 10% of patients in the D stage survive more than a year with an average survival of 3-4 mo[26].

In conclusion, age $\geqslant$ 46 years, alcohol habit and the *MTR A2756G*, *MTHFR A1298C* and *MTRR A66G* polymorphisms are associated with an increased risk of HCC development; age $\geqslant$ 46 years, tobacco habit and the *MTR A2756G* polymorphism are associated with cirrhosis development. There is an interaction between the *MTRR A66G* polymorphism and tobacco consumers with liver cirrhosis. The present study can collaborate to establish the etiologic factors related to HCC and cirrhosis development and to contribute to strategies related to health care.

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COMMENTS

Background

Age ⩾ 46 years, alcohol habit and the *MTR A2756G*, *MTHFR A1298C* and *MTRR A66G* polymorphisms are associated with an increased risk of hepatocellular carcinoma (HCC) development; age ⩾ 46 years, tobacco habit and the *MTR A2756G* polymorphism are associated with cirrhosis development.

Research frontiers

It is already known that polymorphisms cause DNA hypomethylation, which cause abnormal changes in gene expression inactivating suppressor genes tumor.

Innovations and breakthroughs

The authors confirm the literature data that report a positive association between the presence of polymorphisms and consumption of alcohol and tobacco to the development in an cirrhosis and later HCC.

Applications

These results may offer new possibilities of diagnosis with early initiation of treatment reflecting the improved quality of life.

Peer-review

This is a good descriptive study in which the authors analysed patients with cirrhosis, HCC and healthy individuals in the 2013-2015 period, the authors evaluated the association of the risk factors and polymorphisms in *MTHFR C677T*, *MTHFR A1298C*, *MTR A2756G* and *MTRR A66G* genes involved in folate metabolism.

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CASE REPORT

Hepatitis C and double-hit B cell lymphoma successfully treated by antiviral therapy

Giovanni Galati, Lorenzo Rampa, Umberto Vespasiani-Gentilucci, Mirella Marino, Francesco Pisani, Carlo Cota, Alessandro Guidi, Antonio Picardi

Giovanni Galati, Lorenzo Rampa, Umberto Vespasiani-Gentilucci, Alessandro Guidi, Antonio Picardi, Unit of Internal Medicine and Hepatology, Department of Medicine, Università Campus Bio-Medico di Roma, 00128 Rome, Italy

Mirella Marino, Department of Pathology, Regina Elena National Cancer Institute, 00144 Rome, Italy

Francesco Pisani, Hematology and Stem Cell Transplant Unit, Regina Elena National Cancer Institute, 00144 Rome, Italy

Carlo Cota, Dermopahology Unit, San Gallicano Dermatological Institute, IRCCS, 00144 Rome, Italy

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Correspondence to: Giovanni Galati, MD, Unit of Internal Medicine and Hepatology, Department of Medicine, Università Campus Bio-Medico di Roma, via Álvaro del Portillo 21, 00128 Rome, Italy. g.galati@unicampus.it

Telephone: +39-062-25411446
Fax: +39-062-25411944

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Abstract

B cells lymphoma is one of the most challenging extra-hepatic manifestations of hepatitis C virus (HCV). Recently, a new kind of B-cell lymphoma, named double-hit B (DHL), was characterized with an aggressive clinical course whereas a potential association with HCV was not investigated. The new antiviral direct agents (DAAs) against HCV are effective and curative in the majority of HCV infections. We report the first case, to our knowledge, of DHL and HCV-infection successfully treated by new DAAs. According to our experience, a DHL must be suspected in case of HCV-related lymphoma, and an early diagnosis could direct towards a different hematological management because a worse prognosis might be expected. A possible effect of DAAs on DHL regression should be investigated, but eradicating HCV would avoid life-threatening reactivation of viral hepatitis during pharmacological immunosuppression in onco-haematological diseases.

Key words: Hepatitis C; Lymphoma; Direct antiviral agents; Double hit lymphoma; Chronic hepatitis C

Core tip: B cells lymphoma is one of the most chall-

enging extra-hepatic manifestations of hepatitis C virus. Recently, a new kind of B-cell lymphoma, named Double-hit B, was characterized with an aggressive clinical course. This is the first case described in literature of double hit lymphoma with co-existing chronic hepatitis C, successfully treated by new direct antiviral therapies. This case suggests a potential favorable effect of the new antiviral therapies on double hit lymphoma regression.

Galati G, Rampa L, Vespasiani-Gentilucci U, Marino M, Pisani F, Cota C, Guidi A, Picardi A. Hepatitis C and double-hit B cell lymphoma successfully treated by antiviral therapy. *World J Hepatol* 2016; 8(29): 1244-1250 Available from: URL: http://www.wjgnet.com/1948-5182/full/v8/i29/1244.htm DOI: http://dx.doi.org/10.4254/wjh.v8.i29.1244

INTRODUCTION

Hepatitis C virus (HCV) is a major global public health problem, although the newest and most effective antiviral therapies (AVTs), free from severe side effects related to interferon (IFN), are spreading in developed countries. This could lead in the next future to a lower prevalence of chronic hepatitis C (CHC) and of its extra-hepatic manifestations. Among these, the hematologic manifestations are the most challenging. Indeed, HCV could induce B cell proliferation and cause mixed cryoglobulinaemia and non-Hodgkin lymphoma (NHL)[1]. The typical HCV-related lymphomas are marginal zone lymphoma (MZL) and diffuse large B cell lymphoma (DLBCL)[2]. The first could benefit from AVTs, whereas the second group needs more aggressive chemotherapies and the only AVTs seem to be ineffective. More recently, a new kind of lymphoma named double-hit B cell (DHL) was described, characterized by chromosomal rearrangements, specifically of *Myc* oncogene and either B cell lymphoma 2 and B cell lymphoma 6 oncogenes (*Bcl2-Bcl6*) or gene for Cyclin D1 (*Ccnd1*)[3,4]. DHL represents about 5% of all cases of DLBCL and affected patients generally have an aggressive clinical course with poor prognosis, despite combination chemotherapy, with a median overall survival of less than 1-2 years. To date, due to the limited literature concerning this type of lymphoma, the specific treatment is still not clear. A potential correlation between HCV and DHL has never been investigated.

We describe the first case, to our knowledge, of a patient affected by DHL and CHC who underwent a successful antiviral treatment with IFN-free therapy (ombitasvir/paritaprevir/ritonavir and dasabuvir).

CASE REPORT

The patient was a 39-year-old Caucasian woman, who tested HCV positive in 2013 during a routine medical evaluation. She had not history of blood transfusions or other risk factors for hepatitis virus transmission. The CHC was sustained by genotype 1b virus, with low necro-inflammatory activity and mild liver fibrosis according to non-invasive evaluation performed by Fibroscan® (4.5 kPa), whereas HCV showed a high replication rate (4500000 UI/mL, Cobas AmpliPrep/Cobas TaqMan®-Roche, Rotkreuz, Switzerland). Ultrasound scans of the liver did not demonstrate signs of fibrosis or spleen enlargement secondary to portal hypertension. Of interest, the patient reported a previous episode of major depression occurred in 2006 and, since then, she had been taking venlafazine 75 mg/d.

In July 2013, due to the enlargement of neck lymph nodes, the patient underwent a hematological evaluation and a lymph node biopsy, and she was finally diagnosed with DLBCL (CD20+, CD30-, *Bcl2*+, Ki67 50%, *Bcl6* + 25%, negativity for CD10 and *Mum1*), with involvement of nodes on both sides of the diaphragm, bone marrow and spleen (Figure 1). Treatment with cyclophosphamide, doxorubicin, vincristine, prednisolone plus Rituximab (R) (*R-CHOP* regimen) plus prophylactic intrathecal injection of methotrexate and prednisone, was started. In December 2013, *i.e.,* after six courses of *R-CHOP*, the patient showed a complete remission of lymphoma according both to Contrast Tomography plus Positron Emission Tomography scans and to bone marrow biopsy. Two additional doses of R were prescribed and, at that time, blood tests showed normal levels of transaminases, while HCV replication did not show any substantial changes (Figure 2). In the following months, the patient was referred to our Liver Unit and she was evaluated for AVT. However, since "all-oral" AVTs for CHC was at that time unavailable in Italy, the patient was evaluated for IFN-based AVT, but she presented relative and absolute contraindications to IFN use, such as a previous episode of major depression, mild anemia, leucopenia (*i.e.,* Haemoglobin 10.7 g/dL, White cells counts 1.980 cells/mm^3), as well as to ribavirin (RBV) use (anemia). We therefore asked our case to be evaluated for the expanded access program (EAP) which was ongoing with Paritaprevir (PTV), a NS3/4A protease inhibitor co-formulated with NS5A inhibitor Ombitasvir (OBV) and the pharmacokinetic enhancer ritonavir (r), plus non-nucleoside polymerase inhibitor Dasabuvir (DSV). Since lymphoma is a possible extra-hepatic manifestation of HCV, the pharmaceutical industry committee accepted our patient for participation to the EAP. Unfortunately, in October 2014, the patient experienced the first relapse of lymphoma at the right maxillary sinus (Figure 3), whereas bilateral bone biopsies were negative. Since chemotherapy was urgent, cisplatin, cytarabin, dexamethasone plus R (*R-DHAP* regimen) were started. After the second cycle of *R-DHAP*, liver tests worsened, with a rapid evolution to liver failure, *i.e.*, jaundice and ascites (Figure 2). She was admitted to our Unit and the liver decompensation was successfully treated with diuretics and anti-oxidant infusion (glutathione 2 g/d until recovery).

From February 2015 to March 2015 she underwent local radiotherapy on the right maxillary sinus (30 Gray).

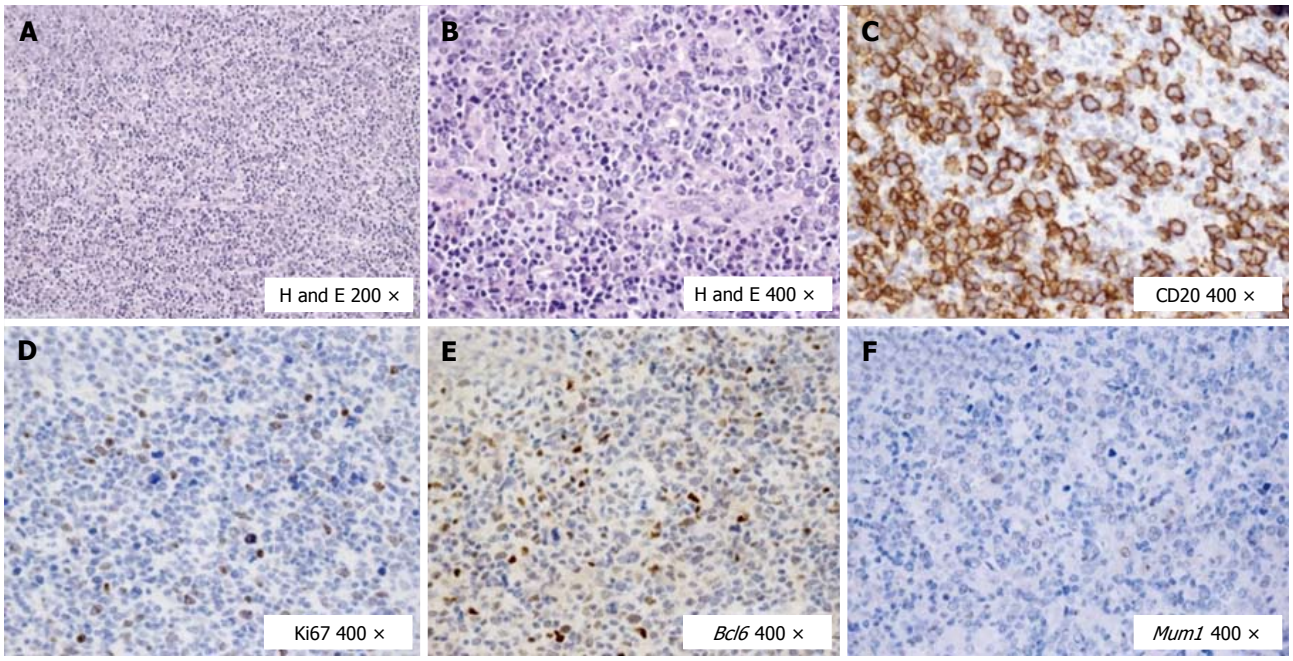

Figure 1 Histological and immunohistochemical features of the diffuse large B cell lymphoma at the primary diagnosis. A: In the lymph node a polymorphic lymphoid population is seen; B: Large lymphoid cells prevailed; C: These large cells were B CD20+; D: The proliferation rate marked by Ki67 was around 20% with respect to the overall population, but about 40% if compared with the blastic B cell population; E: The large B cells expressed mainly the germinal center marker *Bcl6*; F: *Mum1* was not found expressed at the primary diagnosis. DLBCL: Diffuse large B cell lymphoma; H and E: Hematoxylin and eosin; CD: Clusters of differentiation; Ki67: Cellular marker for proliferation; *Bcl6*: B cell lymphoma 6; *Mum1*: Multiple myeloma oncogene 1.

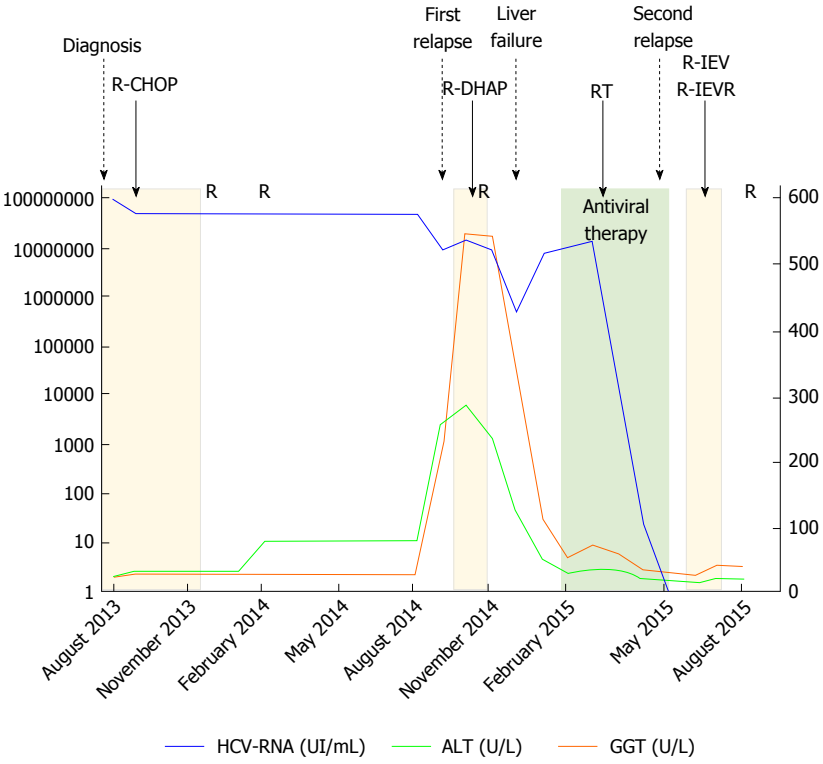

Figure 2 This figure shows the trends of gamma-glutamyl transpeptidase, alanine amino-transferase and hepatitis C viremia - RNA during chemo-therapies and antiviral therapy. R: Rituximab; R-CHOP: Cyclophosphamide, doxorubicin, vincristine, prednisolone plus Rituximab; R-DHAP: Cisplatin, cytarabin, dexamethasone plus Rituximab; R-IEV: Ifosfamide, etoposide, epirubicin plus Rituximab; GGT: Gamma-glutamyl transpeptidase; ALT: Alanine amino-transferase; HCV: Hepatitis C viremia.

After the liver tests returned to normal levels, she started the triple DAAs regimen in the EAP, for a duration of 12 wk (Figure 2). The HCV-RNA showed a fast drop since after two weeks of AVT (42 UI/mL), while viremia

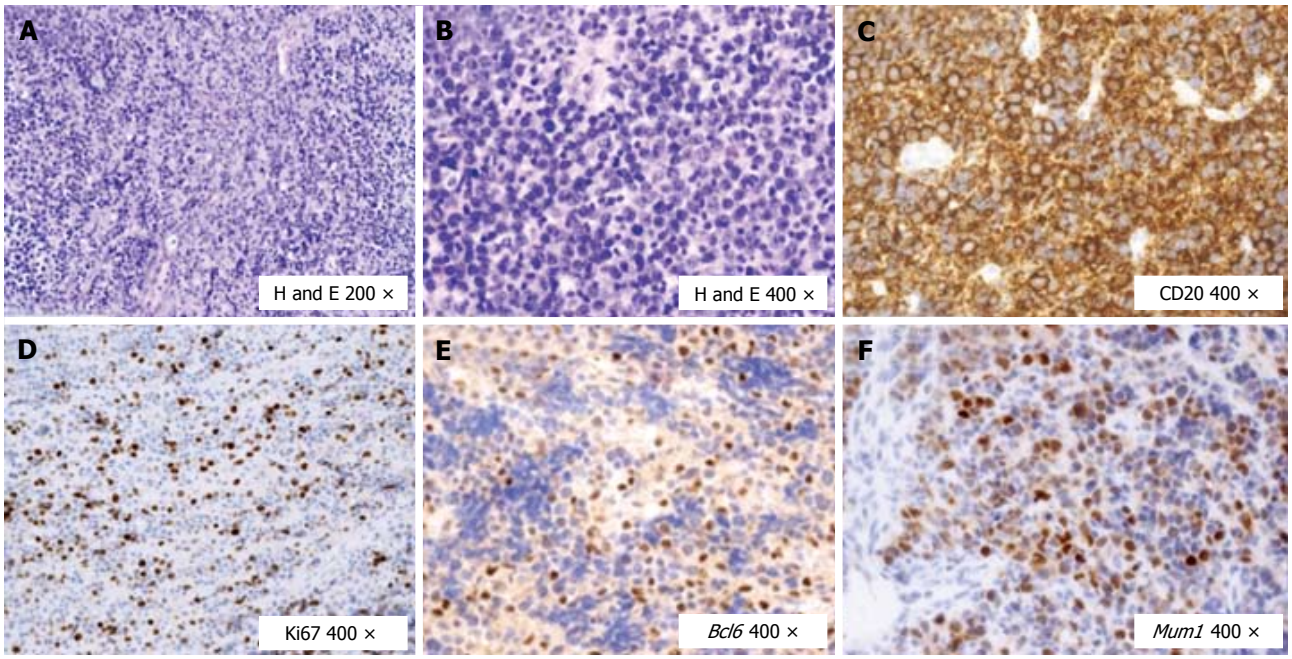

Figure 3 Histological and immunohistochemical features of the diffuse large B cell lymphoma at the first relapse (mucosal). A: In the mucosal relapse a polymorphic lymphoid population was found; B: The rate of large lymphoid cells increased up to 80%; C: These large cells were B CD20+; D: The proliferation rate marked by Ki67 was around 50%-60% with respect to the blastic B cell population; E: The large B cells expressed the germinal center marker *Bcl6* (about 50%-60%); F: *Mum1* was found to be expressed now by about 70% of the cells. DLBCL: Diffuse large B cell lymphoma; H and E: Hematoxylin and eosin; CD: Clusters of differentiation; Ki67: Cellular marker for proliferation; *Bcl6*: B Cell lymphoma 6; *Mum1*: Multiple myeloma oncogene 1.

became undetectable (limit of quantification according to our virological test was 15 UI/mL) after 8 wk of therapy, and persisted undetectable both at the end of AVT and 24 wk after. However, in May 2015, *i.e.,* after 4 wk from the end of AVT, the patient experienced a new relapse of lymphoma (Figure 4). Due to the appearance of multiple cutaneous nodules on the middle third of the left arm, on the posterior region of the chest wall and on the right and left flanks, a biopsy of one of them was indicated, showing a dense lymphoid infiltrate with atypical growth pattern and cells of large size (CD20+, CD79a+, CD5+, Bcl2+, CD3-, CD30-, *Bcl6*-, *Mum1*-, Ki67 80%). A new PET scan showed multiple pathological uptake of fluorodeoxyglucose in inguinal regions and at the level of the subcutaneous nodules, while the bone marrow biopsy did not show any infiltration. Consistent with the history of two previous relapses and to the high expression of *Bcl2*, a histological reassessment was required on both the lymphoid tissue available at diagnosis and that from the biopsy of the subcutaneous nodule. An analysis with fluorescence *in situ* hybridization for the detection of lymphoma-associated chromosomal abnormalities was performed (Vysis LSI *Bcl2* break apart probe, Vysis LSI Myc break apart probe), showing additive copies of *Bcl2* (18q21) in 82% and rearrangement of *Myc* in 89% of the nuclei analyzed (8q24), respectively. Therefore, the final diagnosis was that of DHL, a more aggressive subtype of DBCL. In July 2015, the patient was firstly prescribed a cycle of ifosfamide, etoposide, epirubicin plus R (*R-IEV* regimen), followed by stem cell collection for a possible autologous stem cell transplantation; subsequently, she underwent a second cycle of *R/IEV/R* (regimen with an additive administration of R), which was burdened by infective complications requiring hospitalization and prolonged antibiotic therapies. At the last hepatological follow-up visit in November 2015, *i.e.,* 24 wk after the end of AVT, HCV-RNA was permanently undetectable despite the immunosuppressive therapy. Afterwards, the patient was evaluated for an allogenic stem cells transplantation, which was made in another Hospital in January 2016, and she is free from lymphoma recurrence according to recent clinical and radiological evaluations.

DISCUSSION

The role of HCV in pathogenesis of NHL is well established. The regression of HCV-associated indolent lymphomas after a successful AVT adds the demonstration that there is a relationship between HCV and lymphomagenesis, but there are no data about the response of DHL to AVTs. Because HCV is not integrated into the host genome, there should be indirect mechanisms to induce malignancy ("hit and run theory"). The main hypothesis links the antigenic stimulation caused by CHC to the chronic proliferation of B cells, to produce firstly a polyclonal and after a monoclonal expansion of these cells resulting, in conjunction with further occurrence of additional genetic mutations, in an overt NHL[5]. Notably, several years have to pass in order to accumulate mutational changes, whereas a genetic predisposition or additional mutagenic effects in genes *Bcl2*, *Bcl6* or *Ccnd1*

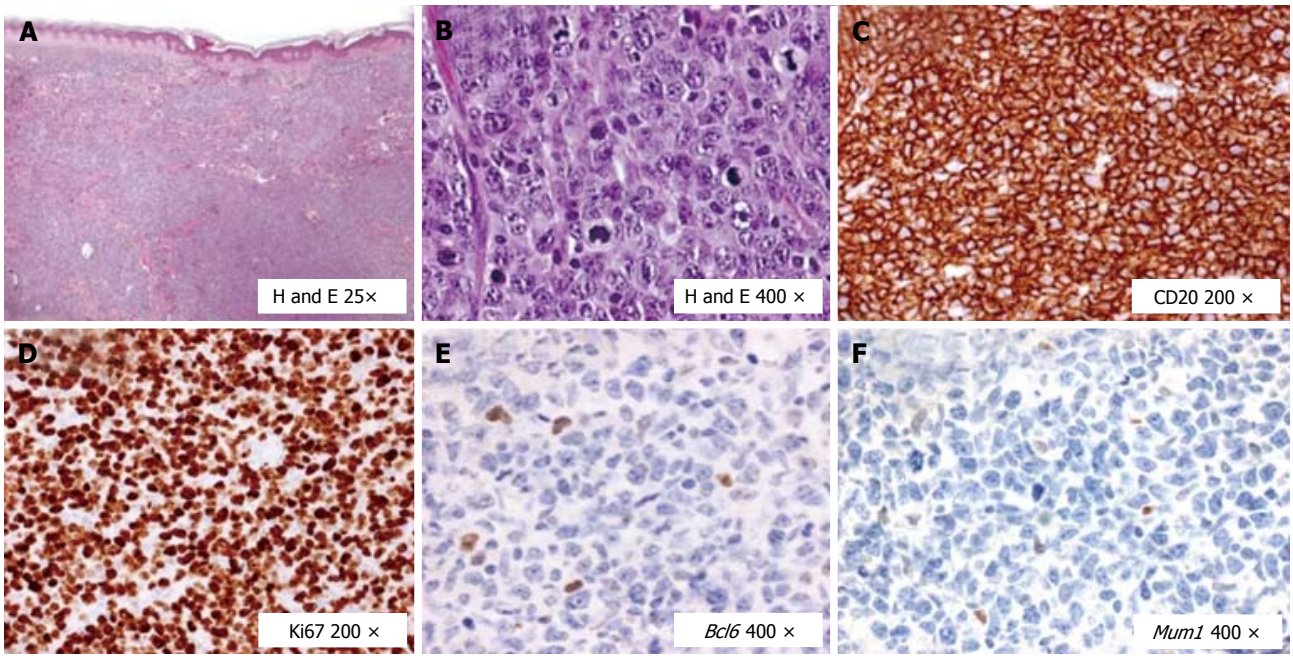

Figure 4 Histological and immunohistochemical features of the diffuse large B cell lymphoma in the second relapse (cutaneous). A: In the cutaneous relapse a rather monomorphic lymphoid population was found; B: The rate of large lymphoid cells increased up to 100%; C: These large cells were B CD20+; D: The proliferation rate marked by Ki67 was around 95% in the blastic B cell population; E: The large B cells expressed poorly the germinal center marker *Bcl6*; F: *Mum1* was found to be poorly expressed by the large B cells. DLBCL: Diffuse large B cell lymphoma; H and E: Hematoxylin and eosin; CD: Clusters of differentiation; Ki67: Cellular marker for proliferation; *Bcl6*: B Cell lymphoma 6; *Mum1*: Multiple myeloma oncogene 1.

could explain the DHL mutation occurrence. Indeed, HCV could induce a mutator phenotype, which involves enhanced mutations of many somatic genes. In the management of HCV-related DLBCL, anthracycline-based chemotherapy, usually *CHOP*, associated with R (chimeric anti-CD20 monoclonal antibody) is the standard of care[6]. Unlike indolent B-cell lymphomas[7], AVT does not play a significant role in HCV-positive DLBCL. Sequential immune-chemotherapy followed by ATV has been used in two studies with promising results leading to improved clinical outcome and prolonged disease free survival[8,9]. On the other hand, a French study reported a higher overall survival and progression free survival in patients treated firstly by AVT, followed in some cases by chemotherapy[10]. All these studies are developed in the "IFN era", both alone or in combination with RBV and, more recently, with protease inhibitors like telaprevir and boceprevir. An IFN-based immunomodulatory and anti-angiogenetic mechanism could be supposed, although IFN-based AVTs cannot be started in most cases, because of contraindications and heavy side effects.

A few reports demonstrated lymphoma regression after HCV clearance with new DAAs. Rossotti *et al*[11] reported a rapid virological and hematological response with the combination of a NS3/NS4A inhibitor (faldaprevir) and a non-nucleoside NS5B inhibitor (deleobuvir) in a patient with HCV associated splenic marginal zone lymphoma. In another report, Sultanik *et al*[12] showed complete regression of MZL after 12 wk of therapy with sofosbuvir (an NS5B RNA-dependent RNA polymerase inhibitor) and RBV. In a case series from France of five patients with HCV-related lymphoma, two DLBCL were successfully treated with sofosbuvir and daclatasvir (an NS5A protease inhibitor), with complete hematological response after 6 mo[13]. There are no reported cases of DHL and CHC treated by new DAAs.

According to clinical data, we could exclude an advanced liver fibrosis in our patient and we estimated a minimal risk of liver decompensation. Therefore, the patient was firstly successfully treated with *R-CHOP*, but after the first hematological relapse, a new immunosuppressive therapy with *R-DHAP* schedule caused an overt liver failure. R has been described to enhance viral replication due to the immune system imbalance, but the rate of severe hepatic complications remains low, with some exceptions, such as in presence of hepatitis B virus or HIV infection, cirrhosis or hepatocellular carcinoma[7,14]. There are controversial studies about the impairment of liver function, which could be caused both by a toxicity of immune-chemotherapy treatment and by HCV reactivation. Indeed, it is not clear if the anecdotal episodes of liver failure in this setting are due to higher HCV replication and enhanced necro-inflammatory activity, or to a direct chemo-toxicity. Chemotherapy-induced HCV reactivation in DLBCL is rarely reported, whereas there are no data about HCV and DHL[15-17]. In our case the liver failure may have been caused by an add-on effect of HCV reactivation and liver chemo-toxicity, as well suggested by a higher increase of Gamma-Glutamyl Transpeptidase, marker of liver toxicity, instead of a pure elevation of Alanine Aminotransferase, a possible expression of an inflammatory response

HCV-mediated. Interestingly, during the liver failure HCV-RNA slightly dropped, so we can suppose that the event was caused by immune re-activation rather than by HCV direct effect on the necro-inflammatory activity. Following the second immune-chemotherapy and after recovering from the acute liver failure, the patient started antiviral therapy with second generation DAAs: OBV/PTV/r and DSV. This multi-targeted 3-DAAs regimen in combination with or without RBV is approved in many countries to treat HCV genotype 1-4 infection. Approval for the treatment of HCV genotype 1 patients with compensated cirrhosis was based on the evidence of a phase 3 trial of 380 patients in which OBV/PTV/r and DSV plus RBV achieved SVR rates at post-treatment week 12 (SVR12) of 91.8% and 95.9% after 12 or 24 wk of therapy, respectively[18]. In the past, the AVTs for HCV were conceived with IFN-based regimens, so an immunomodulatory effect due to interactions with both the adaptive and innate immune response of the host, further than an anti-inflammatory and antiviral effect by inhibiting the synthesis of various cytokines, were possibly responsible for the remission of HCV-related lymphomas. In this context, at the moment, there are no definitive data about IFN free therapies, and it remains to be solved whether IFN is crucial for its direct antitumor and anti-proliferative effect against NHL further than for its antiviral effect.

In conclusion, this is the first case, to our knowledge, of DHL and CHC successfully treated by new DAAs. According to our experience, DHL must be suspected in case of HCV-related lymphoma, and an early diagnosis could direct towards a different hematological management, because a poor prognosis should be expected. Moreover, DAAs triple regimen with OBV/PTV/r and DSV achieves a rapid virological response, and the result is sustained during immunosuppressive therapy, without evidence of HCV reactivation. Finally, we can suggest that in cases of aggressive HCV-related lymphoma, it is mandatory to treat the HCV infection with the new IFN-free regimens at least after the first chemotherapy cycle. This choice could avoid a liver failure in case of re-treatment with hepatotoxic drugs, and it allows a better and safer management of hematological disease. A potential favorable effect of the AVTs on DHL regression should be investigated.

ACKNOWLEDGMENTS

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COMMENTS

Case characteristics

A 39-year-old Caucasian woman presented with neck lymph nodes enlargement and hepatitis C virus (HCV)-infection.

Clinical diagnosis

Painless swelling in the right side of the neck with increased thickness.

Differential diagnosis

Inflammatory process of the throat, metastatic cancer of head-neck sites, lymphoproliferative disease.

Laboratory diagnosis

All blood tests were within normal limits except for high HCV-viremia.

Imaging diagnosis

Computed tomography showed multiple lymph nodes on both sides of the diaphragm.

Pathological diagnosis

Double-hit B cell lymphoma.

Treatments

Chemotherapy, radiotherapy and antiviral therapy for HCV.

Related reports

Double-hit B cell lymphoma is a rare entity with no standardized chemotherapeutic strategies and its association with HCV-infection is not ever been investigated. There are no reports about the efficacy of antiviral therapy for HCV plus chemotherapies, in order to cure this pathology in presence of HCV.

Terms explanation

HCV could induce B cell proliferation and cause non-Hodgkin lymphoma. The typical HCV-related lymphomas are marginal zone lymphoma and diffuse large B cell lymphoma. Double-hit B cell (DHL) is a new kind of lymphoma with more aggressive outcome, and it is characterized by chromosomal rearrangements, specifically of *Myc* oncogene and either B cell lymphoma 2 and B cell lymphoma 6 oncogenes or gene for Cyclin D1.

Experiences and lessons

DHL must be suspected in case of HCV-related lymphoma, and an early diagnosis could direct towards a different hematological management, because a poor prognosis should be expected. A potential favorable effect of new antiviral therapies on DHL regression should be investigated.

Peer-review

This manuscript has reported the first case of DHL and CHC successfully treated by new DAA.

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