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World Journal of Hepatology (*World J Hepatol*, *WJH*, online ISSN 1948-5182, DOI: 10.4254), is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

WJH covers topics concerning liver biology/pathology, cirrhosis and its complications, liver fibrosis, liver failure, portal hypertension, hepatitis B and C and inflammatory disorders, steatohepatitis and metabolic liver disease, hepatocellular carcinoma, biliary tract disease, autoimmune disease, cholestatic and biliary disease, transplantation, genetics, epidemiology, microbiology, molecular and cell biology, nutrition, geriatric and pediatric hepatology, diagnosis and screening, endoscopy, imaging, and advanced technology. Priority publication will be given to articles concerning diagnosis and treatment of hepatology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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REVIEW

Mechanisms of resistance to sorafenib and the corresponding strategies in hepatocellular carcinoma

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Abstract

Sorafenib, the unique drug as first-line treatment for advanced hepatocellular carcinoma (HCC), has opened a window of hope after searching for effective agents to combat HCC for decades. However, the overall outcomes are far from satisfactory. One of the explanations is the genetic heterogeneity of HCC, which has led to identifying predictive biomarkers for primary resistance to sorafenib, and then applying the concept of personalized medicine, or seeking therapeutic strategies such as combining sorafenib with other anticancer agents. Some of the combinations have demonstrated a better effectiveness than sorafenib alone, with good tolerance. The acquired resistance to sorafenib has also drawn attention. As a multikinase inhibitor, sorafenib targets several cellular signaling pathways but simultaneously or sequentially the addiction switches and compensatory pathways are activated. Several mechanisms are involved in the acquired resistance to sorafenib, such as crosstalks involving PI3K/Akt and JAK-STAT pathways, hypoxia-inducible pathways, epithelial-mesenchymal transition, *etc*. Based on the investigated mechanisms, some other molecular targeted drugs have been applied as second-line treatment for treat HCC after the failure of sorafenib therapy and more are under evaluation in clinical trials. However, the exact mechanisms accounting for sorafenib resistance remains unclear. Further investigation on the crosstalk and relationship of associated pathways will better our understanding of the mechanisms and help to find effective strategies for overcoming sorafenib resistance in HCC.

Key words: Hepatocellular carcinoma; Sorafenib; Drug resistance; Cellular signaling pathway; Clinical trials

Core tip: The primary resistance of hepatocellular carcinoma (HCC) to sorafenib is due to genetic heterogeneity. Thus, seeking predictive biomarkers and combining sorafenib with other anticancer agents for HCC have been launched with varying degrees of success. Sorafenib inhibits several kinase targets but it can also simultaneously or sequentially activate the addiction switches and compensatory pathways, inducing acquired resistance. Some other molecular targeted drugs have been used as second-line treatment for advanced HCC after the failure of sorafenib therapy. Further investigation on the crosstalk and relationship of associated pathways will better our understanding of the mechanisms accounting for sorafenib resistance in HCC.

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INTRODUCTION

Liver cancer is the second most frequent cause of cancer

death in men worldwide and hepatocellular carcinoma (HCC) accounts for 70%-85% of the total liver cancer burden[1]. Many lines of clinical investigation indicate that none of the adjuvant therapies is particularly effective in treating HCC after surgery and systemic traditional chemotherapy has a very low response rate for HCC. Recently emerging molecular targeted drugs (MTD) have been demonstrated to be promising agents in prolonging the overall survival (OS) of late stage HCC patients. Particularly, sorafenib has been uniquely recommended as the first line treatment for advanced HCC[2]. Despite the encouraging achievement, the worry about drug resistance to sorafenib is increasing as the OS of HCC patients after sorafenib treatment was only 2-3 mo longer than placebo and sorafenib was shown to result in a limited increase in median time to symptomatic progression and a low partial response rate due to drug resistance[3,4]. Although the exact rate of resistance to sorafenib has not been reported, considering the dilemma that no effective systemic therapy is available so far for patients after failure of sorafenib therapy, studies on the mechanisms of sorafenib resistance are urgently required[5-7]. The present article aims to review the latest progress in this field by focusing on the mechanisms of resistance to sorafenib and the strategies in HCC.

PREDICTION OF SORAFENIB SENSITIVITY

Due to genetic heterogeneity, some HCC cells are initially resistant to sorafenib, which is termed primary resistance[8]. The IC_{50} values of growth inhibition of different HCC cell lines by sorafenib *in vitro* showed big variations[9,10]. Thus, it is important to identify predictive biomarkers for primary resistance to sorafenib.

The activation of RAF/mitogen-activated protein kinase (MAPK)/extracellular signaling-regulated kinase (ERK) signal pathway is commonly observed in HCC[11]. Sorafenib executes its anti-tumor activity partially through targeting the Raf-1 and B-Raf, thus inhibiting the RAF/MEK/ERK signaling pathways. It was reported that sorafenib inhibited the phosphorylated ERK (pERK) in HCC PLC/PRF/5 and HepG2 cells[9]. Zhang *et al*[12] reported that the effects of sorafenib on cell proliferation were significantly correlated with basal pERK levels and the U0126, a selective inhibitor of ERK1/2, could reduce the sensitivity of HCC cells to sorafenib through downregulation of pERK. In a phase Ⅱ clinical study of sorafenib, the pERK levels in tumor samples from 33 patients showed the correlation with median time to progress (TTP)[13]. However, the relationship was not validated in the phase Ⅲ trial[14]. It has recently been reported that the c-Jun N-terminal kinase (JNK), another member of MAPK family, can serve as a biomarker to predict the sensitivity to sorafenib[15]. Hagiwara *et al*[15] examined the JNK activity in 39 tumor specimens from advanced HCC before sorafenib treatment and found that the tumors from the non-responder group had higher expression of phospho-c-Jun and JNK activity. Moreover, the JNK activation correlated with decreased TTP and poor OS. A recent study on patients enrolled in the SHARP trial (the phase Ⅲ, randomized, controlled Sorafenib HCC Assessment Randomized Protocol) investigated predictive biomarkers to sorafenib and showed that the angiogenesis biomarkers Ang2 and VEGF, among ten assessed plasma biomarkers, were independent predictors of the survival of advanced HCC patients. Although the patients with higher soluble c-KIT or lower hepatocyte growth factor (HGF) in sera at baseline showed enhanced survival benefit, neither of them predicted the response to sorafenib[16].

The current available data indicate that candidate biomarkers for sorafenib sensitivity are still of uncertain value. Well-designed prospective clinical studies are required to judge their exact roles in predicting the primary resistance to sorafenib in HCC. In addition, more preclinical studies are also needed to clarify whether the currently known biomarkers are the downstream events of the latent key biomarkers or if these biomarkers vary in individual patients.

MECHANISMS OF ACQUIRED RESISTANCE TO SORAFENIB

Long-term exposure to antitumor drugs often results in reduced sensitivity of the tumor cells to the drug, leading to acquired resistance. Many mechanisms account for acquired resistance to antitumor drugs, such as addiction switching, compensatory pathway because of pathway loops or crosstalk, epithelial-mesenchymal transition (EMT), cancer stem cells, disabling of pro-apoptotic signals, hypoxic microenvironment, *etc*[17-19]. Recently, some studies have also indicated the correlation between these mechanisms and resistance to sorafenib in HCC.

PI3K/Akt pathway and sorafenib resistance

The phosphatidylinositol 3-kinase (PI3K)/Akt and MAPK pathways are the most critical pathways involved in the development and progression of HCC and are activated or overexpressed in a high proportion of HCC tissues. The parallel PI3K/Akt pathway remains unscathed when sorafenib targets the MAPK pathway and tyrosine kinases by inhibiting vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), Ret and c-kit[3]. Considering the existing crosstalk between the PI3K/Akt and MAPK pathways[20], the latent compensatory mechanism of PI3K/Akt pathways in drug resistance to sorafenib has been attracting attention. Sorafenib has been demonstrated to activate Akt and upregulate the phosphorylation of its downstream targets, such as S6K and 4EBP1 in HCC cells[21,22]. A study by Chen *et al*[7] has shown that sorafenib-resistant HCC cells, which were established by long-term exposure to sorafenib, had increased expression of phosphorylated Akt and p85, a regulatory subunit of PI3K, compared with the parental cells. Similarly, the HCC cells with ecto-

pic expression of constitutive Akt also showed resistance to sorafenib. In addition, the resistance to sorafenib could be reversed by gene knockdown of Akt and Akt inhibitor MK-2206. These results indicate that activation of PI3K/Akt pathway may contribute to sorafenib resistance and call for further study in clinical trials.

JAK-STAT pathway and sorafenib resistance

The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway participates in the regulation of cell proliferation, differentiation, survival, motility and apoptosis in many organs, including liver[23,24]. STAT3 plays a critical role in transcriptional regulation of genes and is also activated by many cytokines and growth factor receptors, such as PDGFR, fibroblast growth factor receptor (FGFR) and epidermal growth factor receptor (EGFR) through JAK[25,26]. The negative regulation of STAT3 is mainly executed by suppression of cytokine signaling (SOCS) proteins through JAK and Src-homology protein tyrosine phosphatases (SHPs), such as SHP-1 and SHP-2, and cytokines and growth factor receptors[23]. STAT3 is activated in HCC and knockdown of STAT3 had a therapeutic effect on HCC[27]. It has recently been reported that sorafenib inhibited the activity of STAT3 by downregulating the phosphorylation of STAT3 at the tyrosine and serine site (Y705 and S727) through regulating PI3K/Akt pathway and MAPK pathway, respectively, but had no effect on JAK2 and SHP2 expression[27]. Sorafenib displayed its inhibitory effect on STAT3 in an SHP-1-dependent manner, but not kinase-dependent inactivation of STAT3[28]. Sorafenib also overcomes TRAIL resistance by inhibiting the activation of STAT3 in HCC cells[29]. Several studies have also investigated the role of JAK-STAT pathway in the mechanisms of acquired resistance to sorafenib in HCC. Sorafenib-resistant HCC cells express higher levels of p-STAT3, p-JAK1 and p-JAK2, but lower levels of SHP-1 and p-SHP-1, indicating that the JAK-STAT pathway participates in the acquired resistance to sorafenib in HCC[26]. Interestingly, dovitinib, another multikinase inhibitor targeting VEGFR, FGFR and c-KIT and regulating the JAK-STAT pathway, could reverse the acquired resistance to sorafenib by directly activating SHP-1 and thus downregulating p-STAT3[26]. Inhibition of SHP-1 or gene knockdown of *SHP-1* blocked the effect of dovitinib, indicating that the SHP-1-activating agent may provide second-line treatment after the failure of sorafenib therapy[30].

Hypoxic microenvironment and sorafenib resistance

The hypoxic microenvironment is closely related to the resistance to many antitumor drugs[19]. We have previously demonstrated that targeting hypoxia-inducible pathways enhanced the antitumor activity of doxorubicin in HCC[4,31]. Although sorafenib downregulates the synthesis of hypoxia-inducible factor (HIF)-1α in HCC cells *in vitro* and *in vivo*[32], the correlation of sorafenib resistance and hypoxic microenvironment is attractive because the anti-angiogenic activity of sorafenib is speculated to lead to tumor starvation and subsequent tumor hypoxia[33]. A recent study[34] has shown that sorafenib-resistant HCC tissues had higher expression of HIF-1α than sorafenib-sensitive and pre-treated HCC tissues. In xenograft models, the increased hypoxia because of sustained sorafenib therapy was associated with sorafenib sensitivity. Moreover, EF24, an analogue of curcumin, could synergistically enhance the antitumor effects of sorafenib and overcome sorafenib resistance through inhibiting HIF-1α by sequestering it in cytoplasm and promoting degradation *via* upregulating (von Hippel-Lindau) VHL.

EMT and sorafenib resistance

Epithelial-mesenchymal transition or transformation (EMT) is the transitional phenomenon of epithelial cells to a mesenchymal phenotype which participates in embryonic development and wound healing, and has recently emerged as a pivotal event in the development of the invasive and metastatic potentials of cancer progression, including HCC[35,36]. EMT is regulated by the upstream pathway such as PI3K/Akt pathway, MAPK, *etc*[37]. Emerging evidence suggests that EMT is involved in, and targeting EMT can reverse, the resistance of antitumor drugs[38]. Recently, the role of EMT in the resistance of HCC to sunitinib has been reported[39]. A study showed that sorafenib inhibited the HGF-induced EMT in HCC by downregulating SNAI1 expression *via* the MAPK signaling pathway[37]. The microarray gene expression analysis showed the existence of EMT accompanied by activation of PI3K/Akt and MAPK pathway in sorafenib-resistant HCC cells[40]. The above studies indicate that EMT may be involved in the resistance to sorafenib in HCC but further studies to clarify the specific mechanisms are required.

In addition to the above described mechanisms, some limited studies have also demonstrated that EGFR[10], glucose-regulated protein 78 (GRP78)[41], multidrug resistance protein (MDRP) 2[42], nuclear factor κB (NF-κB)[43,44] and autophagy[45] may be involved in the acquired resistance to sorafenib in HCC.

STRATEGIES FOR OVERCOMING THE RESISTANCE TO SORAFENIB

Although the exact mechanisms of resistance to sorafenib have not yet been fully elucidated, some approaches have been launched to cope with sorafenib resistance in HCC in clinical trials. The completed and ongoing clinical trials for overcoming sorafenib resistance are summarized in Tables 1 and 2, respectively. These trials can be divided into two categories. One is to combine sorafenib with other anticancer drugs and the other is to use other drugs or drug combinations as second-line treatments in HCC patients after the failure of sorafenib therapy.

Combinational therapy with sorafenib

At present, there are dozens of ongoing clinical trials which are evaluating the therapeutic efficacy of sorafenib

Table 1 Completed clinical trials for overcoming sorafenib resistance

Therapeutic strategies	Phases	Cases	Efficacy
Combinational therapy			
5-fluorouracil plus sorafenib[46]	Phase Ⅱ	39	SD: 46.2%; median TTP: 8 mo; OS: 13.7 mo
Tegafur/uracil plus sorafenib[47]	Phase Ⅱ	53	Median PFS: 3.7 mo; median OS: 7.4 mo
Octreotide plus sorafenib[48]	Phase Ⅱ (So.LAR.)	50	SD: 66%; median TTP: 7.0 mo; median OS: 12 mo
Doxorubicin plus sorafenib *vs* doxorubicin plus placebo[50]	Phase Ⅲ	47 *vs* 49	Median TTP: 6.4 mo *vs* 2.8 mo; OS: 13.7 mo *vs* 6.5 mo; PFS: 6.0 mo *vs* 2.7 mo
Erlotinib plus sorafenib *vs* erlotinib plus placebo[53, 54]	Phase Ⅲ (SEARCH)	362	Median TTP: 3.2 mo *vs* 4.0 mo; OS: 9.5 mo *vs* 8.5 mo
Second-line treatments			
Sunitinib[55]	Retrospective analysis	11	SD: 40%; median TTP: 3.2 mo
Brivanib[56]	Phase Ⅱ	46	SD: 41.3%; RR: 4.3%; DCR: 45.7%; median OS: 9.79 mo
Tivantinib *vs* placebo[6]	Phase Ⅱ	71 *vs* 36	Progressive disease: 65% *vs* 72%; TTP: 1.6 mo *vs* 1.4 mo
Gemcitabine plus oxaliplatin[59]	Retrospective analysis	18	Overall RR: 18.8%; SD: 18.8%; median PFS: 3.2 mo; OS: 4.7 mo
Erlotinib plus bevacizumab[61]	Phase Ⅱ	10	No response or SD; median TTP: 1.81 mo; OS: 4.37 mo

SD: Stable disease; TTP: Time to progression; OS: Overall survival; PFS: Progression-free survival; DCS: Disease control rate; RR: Response rate.

Table 2 Ongoing clinical trials for overcoming sorafenib resistance

Studies	Therapeutic strategies	Phases	Primary outcomes
Combinational therapy			
NCT01271504	E7050 plus sorafenib *vs* sorafenib	Phase Ⅱ	Adverse event
NCT01033240	CS-1008 plus sorafenib *vs* sorafenib	Phase Ⅱ	TTP
NCT01539018	Tegafur-uracil plus sorafenib *vs* sorafenib	Phase Ⅱ	TTP
NCT01272557	Doxorubicin plus sorafenib *vs* sorafenib	Phase Ⅱ	TTP
NCT01015833	Doxorubicin plus sorafenib *vs* sorafenib	Phase Ⅲ	OS
NCT01214343	Cisplatin/fluorouracil plus sorafenib *vs* sorafenib	Phase Ⅲ	OS
Second-line treatments			
NCT01507168	GC33 *vs* placebo	Phase Ⅱ	PFS
NCT01273662	Axitinib	Phase Ⅱ	SD
NCT00717756	Lenalidomide	Phase Ⅱ	RR
NCT01545804	Lenalidomide	Phase Ⅱ	SD
NCT01567930	Temsirolimus	Phase Ⅱ	Disease progression
NCT01180959	Erlotinib plus bevacizumab	Phase Ⅱ	PFS
NCT01140347	Ramucirumabplus BSC *vs* placebo plus BSC	Phase Ⅲ	PFS
NCT01108705	Brivanib plus BSC *vs* placebo plus BSC	Phase Ⅲ	OS
NCT00825955	Brivanib plus BSC *vs* placebo plus BSC	Phase Ⅲ	OS
NCT01035229	Everolimus plus BSC *vs* placebo plus BSC	Phase Ⅲ	OS

TTP: Time to progression; OS: Overall survival; SD: Stable disease; PFS: Progression-free survival; RR: Response rate; BSC: Best supportive care.

in combination with other anticancer agents to treat advanced HCC, according to the database of clinical trials from the United States National Institutes of Health (http://www.clinicaltrials.gov). Some completed clinical trials have been promising to some extent by combining sorafenib with other agents.

In a phase Ⅱ trial with 39 advanced HCC patients, sorafenib in combination with 5-fluorouracil infusion showed an encouraging disease control rate with the stable disease (SD) rate of 46.2% for a median duration of 16.2 mo, median TTP of 8 mo and OS of 13.7 mo[46].

Metronomic chemotherapy using tegafur/uracil has been shown to enhance the anti-tumor effect of anti-angiogenic agents in preclinical models. In a phase Ⅱ study with 53 advanced HCC patients, metronomic chemotherapy with tegafur/uracil was safely combined with sorafenib and preliminarily showed the improvement of sorafenib efficacy, with median progression-free survival (PFS) of 3.7 mo and median OS of 7.4 mo[47].

In a multicenter phase Ⅱ So.LAR. study with 50 advanced HCC patients, the combinational therapy with sorafenib and long-acting octreotide resulted in SD rate of 66%, median TTP of 7.0 mo and median OS of 12 mo[48]. The results suggest that the combination between sorafenib and long-acting octreotide is active and well tolerated in patients with advanced HCC and could represent another efficacious chance for the management of this population[48].

Doxorubicin is considered one of the most effective cytotoxic agents and is widely used in the treatment of HCC, especially *via* transcatheter arterial chemoembolization (TACE)[4,49]. In a phase Ⅲ trial, doxorubicin plus sorafenib compared with doxorubicin alone was evaluated in 96 patients with advanced HCC[50]. The sorafenib plus doxorubicin achieved longer median TTP (6.4 mo *vs* 2.8 mo), OS (13.7 mo *vs* 6.5 mo) and PFS (6.0 mo *vs* 2.7 mo) than doxorubicin placebo monotherapy. The only grade 2/3 adverse event of left ventricular dysfunction was seen in one patient in the sorafenib plus doxorubicin group. However, because doxorubicin was used as the

controlled arm in this trial, the encouraging outcome was unable to justify that the efficacy was from sorafenib alone or the synergism with doxorubicin. Now, a randomized phase Ⅲ trial aiming to evaluate the combinational therapy of doxorubicin plus sorafenib compared with sorafenib alone is recruiting participants (ClinicalTrials.gov, NCT01840592).

Erlotinib, an oral tyrosine kinase inhibitor of EGFR, has shown a modest antitumor activity against HCC[51,52]. To evaluate the effect of sorafenib in combination with erlotinib, a randomized, placebo-controlled, double-blind, phase Ⅲ study (SEARCH trial, NCT00901901) is being conducted with sorafenib as the controlled arm. However, the preliminary results reported in the 37th European Society for Medical Oncology (ESMO) Congress[53,54] did not show that the addition of erlotinib to sorafenib met the primary endpoint and the median OS and TTP was not statistically different in the experimental and controlled arms.

Second-line treatments

Many anticancer drugs, most of which are MTDs, such as VEGFR inhibitors (axitinib and ramucirumab), mTOR inhibitors (everolimus and temsirolimus), EGFR inhibitor (erlotinib) in combination with VEGFR inhibitor (bevacizumab) and GC33, a recombinant humanized antibody against glypican-3, are being tested as second-line treatments for advanced HCC in clinical trials (http://www.clinicaltrials.gov).

Sunitinib, a multikinase inhibitor targeting the similar receptors to sorafenib, such as VEGFR, PDGFR and RAF, showed a modest antitumor activity in 11 sorafenib-resistant patients with SD in 40% patients and median TTP of 3.2 mo[55]. Undesirably, sunitinib as second-line treatment did not show the antitumor activity in HCC patients with Child-Pugh class B liver cirrhosis because these patients died within 4 mo due to the clinical deterioration of liver function and tumor progression.

Brivanib, a selective dual inhibitor of FGFR and VEGFR, has shown antitumor activity against HCC[56]. A phase Ⅱ open-label study assessed brivanib as second-line treatment in HCC patients who had failed prior to antiangiogenic treatment, including sorafenib[56]. In 46 enrolled patients, brivanib was administered orally at a dose of 800 mg once daily and the SD, tumor response rate and disease control rate was 41.3%, 4.3% and 45.7%, respectively. The median OS was 9.79 mo. The results show that brivanib may be safe and efficient in treating advanced HCC after sorafenib therapy. However, a press release in July, 2012 from Bristol-Myers Squibb, the manufacturer of brivanib, revealed that brivanib did not meet the primary endpoint of improving overall survival *vs* placebo in the phase Ⅲ trial (http://news.bms.com/press-release/).

Recently, a multicenter, randomized, placebo-controlled, double-blind, phase Ⅱ study (ClinicalTrials.gov, NCT00988741) reported the results of using tivantinib, a selective oral inhibitor of MET, as second-line treatment in sorafenib-resistant HCC[6]. Among the 107 enrolled patients, 104 patients had received sorafenib treatment. Seventy-one patients were randomly assigned to receive tivantinib (38 at 360 mg twice daily and 33 at 240 mg twice daily) and 36 patients to receive placebo. At the time of analysis, 46 (65%) patients in the tivantinib group and 26 (72%) of those in the placebo group had progressive disease. After the median follow-up of 5.5 mo, the tivantinib group had a longer TTP than the placebo group (1.6 mo *vs* 1.4 mo). The 22 (31%) patients with MET-high tumors treated with tivantinib had a median TTP of 2.7 mo, which was significantly longer than that (1.4 mo) for 15 MET-high patients (42%) on placebo. Interestingly, tivantinib at the dose of 240 mg (twice per day) showed slightly longer OS and moderate adverse events compared to the schedule of 360 mg. These results provide an option for second-line treatment of advanced HCC patients, particularly for those with MET-high tumors, after failure of sorafenib and call for further phase Ⅲ trials. The report may also imply that Met might serve as a predictive biomarker in this case.

A drug combination of gemcitabine plus oxaliplatin has shown antitumor activity again HCC[57,58]. Thus, it was used as second-line treatment in HCC patients after sorafenib pretreatment. In a clinical trial with 18 patients after the failure of sorafenib therapy, gemcitabine plus oxaliplatin treatment showed an overall response rate of 18.8%, SD of 18.8%, PFS of the median 3.2 mo and OS of 4.7 mo with moderate adverse events[59].

Erlotinib plus bevacizumab has shown an apparently synergistic effect with acceptable adverse events as first-line treatment of HCC[60]. To evaluate the effects of erlotinib in combination with bevacizumab as second-line therapy after the failure of sorafenib, a phase Ⅱ trial is ongoing (ClinicalTrials.gov, NCT01180959). However, another similar phase Ⅱ trial executed during the same period showed disappointing interim results[61]. Among the ten recruited patients after first-line sorafenib treatment, no response or SD were achieved and the median TTP and OS was 1.81 and 4.37 mo, respectively. Adverse events were common, with rash in 70%, diarrhea in 50% and malaise in 40% of patients. Thus, this trial was halted after the interim analysis[61]. The results of the ongoing similar trial are expected.

CONCLUSION

In summary, the mechanisms accounting for the resistance of HCC to sorafenib are complicated and remain unclear. The primary resistance of HCC to sorafenib is possibly due to the genetic heterogeneity. Seeking predictive biomarkers and therapeutic strategies by combining sorafenib with other anticancer agents have been launched with varying degrees of success. Sorafenib inhibits several kinase targets but it can also simultaneously or sequentially activate the addiction switches and compensatory pathways, such as PI3K/Akt and JAK-STAT pathways, tumor hypoxia, EMT, *etc.*, leading to

acquired resistance. Some other MTDs have been applied as second-line treatment for advanced HCC after the failure of sorafenib therapy and more are under evaluation in clinical trials. Further investigation on the crosstalk and relationship of associated pathways will better our understanding of the mechanisms and effective strategies for overcoming sorafenib resistance in HCC are being sought.

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ORIGINAL ARTICLE

Herbalife hepatotoxicity: Evaluation of cases with positive reexposure tests

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Abstract

AIM: To analyze the validity of applied test criteria and causality assessment methods in assumed Herbalife hepatotoxicity with positive reexposure tests.

METHODS: We searched the Medline database for suspected cases of Herbalife hepatotoxicity and retrieved 53 cases including eight cases with a positive unintentional reexposure and a high causality level for Herbalife. First, analysis of these eight cases focused on the data quality of the positive reexposure cases, requiring a baseline value of alanine aminotransferase (ALT) < 5 upper limit of normal (N) before reexposure, with N as the upper limit of normal, and a doubling of the ALT value at reexposure as compared to the ALT value at baseline prior to reexposure. Second, reported methods to assess causality in the eight cases were evaluated, and then the liver specific Council for International Organizations of Medical Sciences (CIOMS) scale validated for hepatotoxicity cases was used for quantitative causality reevaluation. This scale consists of various specific elements with scores provided through the respective case data, and the sum of the scores yields a causality grading for each individual case of initially suspected hepatotoxicity.

RESULTS: Details of positive reexposure test conditions and their individual results were scattered in virtually all cases, since reexposures were unintentional and allowed only retrospective rather than prospective assessments. In 1/8 cases, criteria for a positive reexposure were fulfilled, whereas in the remaining cases the reexposure test was classified as negative (n = 1), or the data were considered as uninterpretable due to missing information to comply adequately with the criteria (n = 6). In virtually all assessed cases, liver unspecific causality assessment methods were applied rather than a liver specific method such as the CIOMS scale. Using this scale, causality gradings for Herbalife in these eight cases were probable (n = 1), unlikely (n = 4), and excluded (n = 3). Confounding variables included low data quality, alternative diagnoses, poor exclusion of important other causes, and comedication by drugs and herbs in 6/8 cases. More specifically, problems were evident in some cases regarding temporal association, daily doses, exact start and end dates of product use, actual data of laboratory parameters such as ALT, and exact dechallenge characteristics. Shortcomings included scattered exclusion of hepatitis A-C, cytomegalovirus and Epstein Barr virus infection with only globally presented or lacking parameters. Hepatitis E virus infection was considered in one single patient and found positive, infections by herpes simplex

virus and varicella zoster virus were excluded in none.

CONCLUSION: Only one case fulfilled positive reexposure test criteria in initially assumed Herbalife hepatotoxicity, with lower CIOMS based causality gradings for the other cases than hitherto proposed.

Key words: Herbalife hepatotoxicity; Herbalife induced liver injury; Herbal hepatotoxicity; Herb induced liver injury; Herbs

Core tip: Our analysis focuses on published cases of suspected Herbalife hepatotoxicity with positive reexposure tests and high causality gradings. Problems included poorly fulfilled test criteria, numerous confounding variables, and the use of liver unspecific, obsolete causality assessment methods. Submitting the case data to well established criteria for positive reexposure tests, the test was positive in 1/8 cases and negative or uninterpretable in the other cases. Using the liver specific Council for International Organizations of Medical Sciences scale, causality was probable in 1 case, unlikely and excluded in the other cases. Thus, causality levels were much lower than hitherto proposed.

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INTRODUCTION

Considerable interest focused on the question whether few Herbalife products are potentially hepatotoxic like some other herbal products and dietary supplements[1-10]. These reports created safety concerns and led to editorials[11-13], commentaries[14-16], and critical Letters to the Editor[17-27], all addressing relevant issues[11-27]. Speculations about bacterial contamination with *Bacillus subtilis* in Herbalife products emerged[8,12], and potentially hepatotoxic ingredients such as green tea extracts, *ephedra sinica*, aloe, or vitamin A overdose have been proposed as culprits[2-4,10]. In addition, overall case data quality was mixed due to confounding variables, missing firm exclusion of alternative explanations, and the use of problematic causality attribution methods[1-10]. For hepatotoxicity cases, even with stringent causality assessment the culprits remain undetected in up to 38% of severe liver disease[28], and alternative causes are frequently found[16,29,30], with up to 47% in initially assumed drug induced liver injury (DILI) cases[16,29] and with an average of 49% in initially suspected herb induced liver injury (HILI) cases[30].

When adjusted for case duplications, Herbalife hepatotoxicity was suspected in 53 cases[1-10]. Among these were eight cases with high causality gradings for Herbalife products because of positive unintentional reexposure tests, though criteria to evaluate reexposure tests were not presented[1-5]. A positive reexposure test is commonly considered as gold standard to establish causality for hepatotoxicity[1-5,31-35], provided specific and well established criteria are fulfilled[31-34]. A preliminary study revealed that in 17/30 cases of herbal hepatotoxicity with initially positive reexposure tests the presented data did not fulfil core criteria of a positive reexposure test or that the quality of case data was insufficient and led to uninterpretable results[16].

In this study, case data with assumed Herbalife hepatotoxicity and a positive unintentional reexposure test were reevaluated for fulfilment of specific and well established reexposure criteria and for liver specific causality assessments.

MATERIALS AND METHODS

Patients

We searched the Medline database for the terms "Herbalife hepatotoxicity" and "Herbalife induced liver injury" and retrieved ten publications; 53 cases were identified after adjustment for duplications[1-10]. Details were provided in case reports and case series of hepatotoxicity with assumed causal relationship to Herbalife products. In eight patients, a positive reexposure test with Herbalife was reported[1-5] with causality levels of highly probable[1], certain[2,3], likely and certain[4], and definite and probable[5]. These eight cases represented the study group.

Methods

All data sets of the eight patients with suspected Herbalife hepatotoxicity and positive reexposure tests were analyzed for specific criteria to establish a positive test result according to the conclusions of an international consensus meeting[31]. Some prerequisites are necessary to ensure transparency and reproducibility of this method. First, a baseline alanine aminotransferase (ALT) value < 5 upper limit of normal (N) is required after the first exposure and before the reexposure, with N as the upper limit of the normal range. Second, during reexposure the ALT value must be at least doubled as compared to the baseline ALT value before reexposure. Only when both criteria are met, a positive reexposure test can be assumed, otherwise the test is negative; the test is uninterpretable, if required information is not presented. Validated reexposure tests meeting the specific criteria are included in the Council for International Organizations of Medical Sciences (CIOMS) scale[32,34]. Time to onset of increased liver values after reexposure should be 1-15 d rather than ⩾ 16 d, thus providing additional strengths[31,32,34].

Causality assessment methods as reported in the eight Herbalife cases were evaluated in detail. Subsequently, causality was reevaluated using the quantitative, liver specific and structured CIOMS scale validated for hepatotoxicity[32] and its update as algorithm for hepatotoxicity causality assessment[34]. Causal relation to hepatotoxicity

requires ALT and/or alkaline phosphatase (ALP) values to be at least 2 N[32,34]; the type of injury was assessed as described, since a specific damage pattern is essential for further causality assessment[32]. To differentiate between the hepatocellular, cholestatic or mixed hepatocellular-cholestatic type of hepatotoxicity, serum ALT and ALP values are to be evaluated on the day the diagnosis of Herbalife hepatotoxicity was suspected. Each activity is expressed as a multiple of N, and the ratio (R) of ALT/ALP is calculated. Hepatocellular liver injury is assumed if ALT > 2 N with normal ALP, or R $\geqslant$ 5; cholestatic liver injury is assumed if there is an increase of ALP > 2 N with normal ALT or R $\leqslant$ 2; mixed cholestatic-hepatocellular type of liver injury is assumed in all other cases, *i.e.,* ALT > 2 N, ALP is increased with R > 2 and R < 5. Separate CIOMS scales are designed for either the hepatocellular type of liver injury or the cholestatic ($\pm$ hepatocellular) type[32,34].

RESULTS

Characteristics of the study group

The age of the eight patients ranged from 30 to 78 years (average 51 years) (Table 1). The female: male ratio was 7:1. Two patients originated from Switzerland (cases 1 and 5), three patients from Israel (cases 2-4), one patient from Iceland (case 6), and two patients from Spain (cases 7 and 8). Outcome was favourable in all patients. For each individual patient, all available details for the analysis of reexposure tests and causality assessments are listed (Table 1).

Most quality problems with missing data occurred in retrospective case series, and uncertainties to exclude or verify alternative causes remained from nonspecific parameters used. Available data was incomplete regarding case descriptions, daily doses, exact start and end dates of product use, actual values of laboratory parameters such as ALT, and exact dechallenge characteristics (Tables 1 and 2). In some cases, Herbalife consumption was described as "along the manufacturer's recommendations". In none of the cases was the daily dose of the Herbalife product quantified (Tables 1 and 2). Though exact start and end dates of Herbalife intake and onset of symptoms or increased liver values were missing in all cases, time on Herbalife and time to onset was available. Temporal association between Herbalife use and liver disease was present in all but one patient (case 5) (Table 2); in this patient, lack of temporal association results in lack of causal association (Table 1). In addition, actual data of laboratory parameters such as ALT with exact results and dates were rarely provided and raised questions about the dechallenge characteristics (Tables 1 and 2). Finally, comedication by drugs and/or herbs as confounding variable was reported in 6/8 cases (75%) (Table 2), but details about daily doses and duration of comedications were scattered and complicated clear causality attribution to comedication.

Core criteria to confirm or exclude alternative causes rely on abdominal and hepatobiliary tract imaging, but results were scattered and poorly provided in at best five of the eight patients (Tables 1 and 2). Abdominal ultrasound revealed cholecystolithiasis in one patient (case 1); however, imaging conditions were difficult, liver, gall bladder wall, extrahepatic bile ducts, and pancreas were not evaluated in this particular case (Table 1). Abdominal ultrasound was reported as normal and without evidence for non alcoholic fatty liver disease in three patients (cases 2-4), though details of gallbladder, bile ducts, and pancreas were missing (Table 1). In another patient (case 5) with chronic hepatitis E virus (HEV) infection, abdominal ultrasound was probably performed but data were not provided (Table 1). For all patients of this case series, exclusion of obstructive or tumorous liver disease by appropriate imaging techniques was described, usually by ultrasound imaging. In case 6, "tests did not indicate any other liver disease", but no technical details were specified, and extrahepatic causes were not excluded (Table 1). In two additional patients (cases 7 and 8), abdominal ultrasound was not reported (Table 1). Overall, abdominal ultrasound examinations were either poorly documented or lacking in these eight patients, making exclusion of alternative causes difficult.

In virtually none of the eight patients hepatitis A virus (HAV), hepatitis B virus (HBV), and hepatitis C virus (HCV) infections were excluded by specific tests like anti-HAV-immunoglobulin M (IgM), hepatitis B surface antigen, anti-HBc IgM, HBV-DNA, anti-HBc-IgM, anti-HCV, and HCV-RNA (Table 1). However, vague descriptions were provided such as: "the hepatitis serology (HAV, HBV, HCV) gave no clue for an acute viral hepatitis" (case 1); "investigation for causes of liver damage included viral entities (hepatitis A, B, C viruses)" (cases 2-4); "exclusion of hepatitis A, B, C" (case 5); "tests did not indicate other liver diseases" (case 6); no statement regarding viral serology at all (case 7); and "negative viral serology" (case 8) (Table 1). Though confounding variables prevail and uncertainty exists, it was assumed in favour of the reports that hepatitis A, B, and C was excluded to a major extent in cases 1-5 but not in cases 6-8 (Table 2). Hepatitis E virus infection was considered and found in one patient (case 5) but not reported for the remaining seven patients (Tables 1 and 2). Exclusion of cytomegalovirus (CMV) and Epstein Barr virus (EBV) infection without specific parameters was reported in four patients (cases 1-4), and of herpes simplex virus (HSV) and varicella zoster virus (VZV) infection in none (Tables 1 and 2). Thus, these confounding variables are to be considered for causality assessment in assumed Herbalife hepatotoxicity.

Analysis of reexposure tests

Based on the specific criteria of reexposure tests for the hepatocellular type of liver injury (Table 3), the modalities of unintentional reexposure tests have been described and analyzed in detail for all eight cases (Table 4). Criteria for a positive reexposure test were fulfilled for only one patient (case 1), reexposure was negative

Table 1 Clinical data of all eight patients with liver disease and a reported positive reexposure test by Herbalife products

Patient	Identification	Specific information for each individual patient
1	Hoffmann *et al*[1], 63 yr female	Herbalife product of unknown daily dose for several weeks. BMI 30. Intended weight loss of 14 kg within the past 3 mo. Loss of appetite, nausea, vomiting, and abdominal crampy pains for 2 wk prior to first presentation with increasing jaundice, pale stool and dark urine, transient urticarial exanthema. Comedication: hydrochlorothiazide/amiloride for hypertension since 2 yr and celecoxib temporarily for relapsing vertebral pain syndrome. ALT 1897 U/L, AST 2098 U/L, ALP 248 U/L. Upon discontinuation of all drugs and Herbalife, ALT 35 U/L within 2 mo. Four weeks later, recurrent ALT increase with peak ALT 758 U/L under Herbalife reexposure, but duration of use not communicated and clear temporal association not evaluable. Exclusion of acute infection by HAV, HBV, HCV, CMV, and EBV reported, but details of parameters not communicated. HEV, HSV, and VZV not excluded. Pancreatitis not excluded. Slightly increased ANA and AMA. Difficult assessment conditions: abdominal ultrasound showed cholecystolithiasis, but number of stones and exclusion of cholecystitis and bile duct obstruction not reported, and magnetic resonance cholangiography not performed. Liver histology with acute cholestatic hepatitis, inflammatory biliary lesions, confluent necroses, and eosinophilic infiltration. For the first clinical episode, therefore, synthetic drugs, Herbalife, symptomatic cholecystolithiasis with crampy abdominal pains and possible transient choledocholithiasis, or an incipient overlap syndrome may have been responsible; for the second episode, Herbalife, the biliary disease, and an incipient overlap syndrome remain as culprits. For Herbalife, CIOMS 7 points Final diagnosis: Probable Herbalife hepatotoxicity, symptomatic biliary stone disease, or incipient overlap syndrome as less probable alternatives
2	Elinav *et al*[2], their case 1, 55 yr female	Herbalife products of unknown daily dose for 6 mo. BMI 33. Comedication: aspirin, metformin for non-insulin dependent diabetes mellitus, statins for hyperlipidemia. Lack of reported symptoms and actual data of ALT, AST, and ALP values initially and later on. Following first exposure, medications and Herbalife were stopped, resulting in complete recovery without any described details. One month after Herbalife reuse, a second flare of hepatitis was reported without any details, except that steroid treatment was initiated, which modulated the natural course. Together with Herbalife cessation, this resulted in complete recovery. Serology of HAV, HBV, HCV, CMV, and EBV was negative but not further specified and no reported serology for HEV, HSV, and VZV. Normal abdominal ultrasound. For Herbalife, CIOMS 2 points Final diagnosis: Unlikely Herbalife hepatotoxicity
3	Elinav *et al*[2], their case 2, 48 yr female	Herbalife products of unknown daily dose for 9 mo. BMI 32. Comedication: alpha adrenergic blocker for hypertension of unknown daily dose and treatment duration. Symptoms and actual values of ALT, AST, and ALP not reported. Resolving hepatitis following Herbalife cessation, but missing supportive data. A month after discharge reuse of Herbalife with a second episode, but liver values or further details not communicated. Serology of HAV, HBV, HCV, CMV, and EBV was negative but not further specified and no reported serology for HEV, HSV, and EBV. Normal abdominal ultrasound. Liver histology: hepatocellular hepatitis. For Herbalife CIOMS 1 point Final diagnosis: Unlikely Herbalife hepatotoxicity
4	Elinav *et al*[2], their case 12, 78 yr female	Herbalife products of unknown daily dose for 12 mo. BMI 27. Comedication: biphosphonates and aspirin of unknown daily dose and duration, background illness psoriasis and non insulin dependent diabetes mellitus. Lack of reported symptoms and of ALT, AST, and ALP initially and later on. Serology of HAV, HBV, HCV, CMV, and EBV was negative but not further specified and no reported serology for HEV, HSV, and VZV. Normal abdominal ultrasound. A second hepatitis flare developed after Herbalife reuse, but details not provided except that the hepatitis was unresolved at the time of manuscript submission. For Herbalife, CIOMS 2 points Final diagnosis: Unlikely Herbalife hepatotoxicity
5	Schoepfer *et al*[3], their case 1, 30 yr male	Herbalife products for 26 mo according to the manufacturer's recommended dose (exact daily dose not communicated). BMI 33. Painless jaundice as symptom. Reported initial liver enzymes as fold upper limit of normal: ALT 50, AST 19, and ALP 1.8, but lack of actual values in the subsequent course. Lack of any specific parameters and data on HAV, HBV, HCV, CMV, EBV, HSV, and VZV. Data for abdominal ultrasound not reported. Patient recovered from the first episode, but details of ALT values not provided and Herbalife cessation not communicated. At a second episode of jaundice, positive hepatitis E IgG antibodies. Liver histology showed acute hepatitis with dense neutrophilic and lymphocytic infiltration, multiple apoptotic bodies, and discrete endophlebitis of central veins. The pathologist considered these findings compatible with hepatitis E. Histology at a third episode showed also fibrosis and incomplete cirrhosis. Only after this third episode, the patient was advised to stop his intake of Herbalife products. Between the three episodes and around a fourth episode, normalization of ALT has never been documented, nor a real reexposition after a period of Herbalife cessation. Thus, chronic hepatitis E with incomplete cirrhosis and undulating liver values is the more likely diagnosis rather than Herbalife hepatotoxicity. For Herbalife, CIOMS -1 point Final diagnosis: Chronic hepatitis E, excluded Herbalife hepatotoxicity
6	Jóhannsson *et al*[4], their case 4, 44 yr female	Herbalife products of unknown daily dose for 5-6 mo. BMI unknown. Abdominal pain and jaundice as symptoms with a latency period of 4-5 mo. Comedication: bupropion of unknown daily dose for 20 d. ALT 2637 U/L, ALP 231 U/L. After stopping Herbalife and bupropion, normal liver values reported but details and time course not presented. Following Herbalife reuse, rise in liver values without any further details and normalization after 2 mo. Tests did not indicate any other liver disease, but no details described. Poorly documented case. For Herbalife, CIOMS -2 points Final diagnosis: Excluded Herbalife hepatotoxicity
7	Manso *et al*[5], their case 12, 39 yr female	Herbalife products of unknown daily dose for 60 d. Unknown BMI. No comedication. ALT 1200 U/L, AST 394 U/L, and ALP 454 U/L. Hepatitis improved after Herbalife cessation, but details of ALT values and time course not reported. Shortly after Herbalife rechallenge, recurrent increase of ALT with normalization after Herbalife withdrawal, but actual ALT values and time course not presented. No viral serology, no abdominal ultrasound. Insufficiently documented case. For Herbalife, CIOMS 1 point Final diagnosis: Unlikely Herbalife hepatotoxicity

8	Manso *et al*[5], their case 20, 49 yr female	Herbalife products of unknown daily dose for 2 yr. Unknown BMI. Comedication: Bach flowers. ALT 922 U/L, AST 702 U/L, ALP 201 U/L. Upon cessation of Herbalife and Bach flowers, ALT 793 U/L within 21 d. Eight days after Herbalife reintroduction, ALT 1500 U/L with lack of ALT normalization following Herbalife recessation. Negative viral serology reported, but no details presented. Abdominal ultrasound data not reported and obviously not done. Insufficiently documented case. For Herbalife, CIOMS 0 points Final diagnosis: Excluded Herbalife hepatotoxicity

Details are presented for eight patients with liver disease and a published positive reexposure test to Herbalife products. ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AMA: Antimitochondrial antibodies; ANA: Antinuclear antibodies; AST: Aspartate aminotransferase; BMI: Body mass index in kg/m^2; CIOMS: Council for International Organizations of Medical Sciences; CMV: Cytomegalovirus; EBV: Epstein Barr virus; HAV: Hepatitis A virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HEV: Hepatitis E virus; HSV: Herpes simplex virus; VZV: Varicella zoster virus.

Table 2 Overview of known information of eight cases with suspected Herbalife hepatotoxicity and positive reexposure tests

Presented information	Cases	Individual cases
Daily dose	0/8	-
Exact date of Herbalife start	0/8	-
Exact date of Herbalife end	0/8	-
Exact date of symptoms	0/8	-
Time on Herbalife	8/8	1, 2, 3, 4, 5, 6, 7, 8
Time to onset	8/8	1, 2, 3, 4, 5, 6, 7, 8
Temporal association	7/8	1, 2, 3, 4, 6, 7, 8
Specific symptoms	3/8	1, 5, 6
ALT value	5/8	1, 5, 6, 7, 8
AST value	4/8	1, 5, 7, 8
ALP value	5/8	1, 5, 6, 7, 8
ALT dechallenge	3/8	1, 7, 8
ALT normalization	1/8	1
Hepatobiliary tract imaging	5/8	1, 2, 3, 4, 5
HAV	5/8	1, 2, 3, 4, 5
HBV	5/8	1, 2, 3, 4, 5
HCV	5/8	1, 2, 3, 4, 5
HEV	1/8	5
CMV	4/8	1, 2, 3, 4
EBV	4/8	1, 2, 3, 4
HSV	0/8	-
VZV	0/8	-
Drug comedication	5/8	1, 2, 3, 4, 6
Herbal comedication	2/8	6, 8
Liver histology	3/8	1, 3, 5

Data are derived from the eight cases with details described in Table 1. Time to onset indicates time to symptoms, alternatively to abnormal liver tests. Alanine aminotransferase (ALT) dechallenge and ALT normalization refers only to cases with presented actual ALT values. ALP: Alkaline phosphatase; AMA: Antimitochondrial antibodies; ANA: Antinuclear antibodies; AST: Aspartate aminotransferase; BMI: Body mass index in kg/m^2; CIOMS: Council for International Organizations of Medical Sciences; CMV: Cytomegalovirus; EBV: Epstein Barr virus; HAV: Hepatitis A virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HEV: Hepatitis E virus; HSV: Herpes simplex virus; VZV: Varicella zoster virus.

in another patient (case 8) (Table 4). In the remaining six patients (cases 2-7), exact ALT values before and at reexposure were only partially or not at all documented, leaving these cases uninterpretable. Two additional cases were presented with questionable positive reexposure test upon first look (Table 4); analysis showed lack of any evidence for a positive test.

Causality assessment

Liver unspecific causality assessment methods were applied in case 1 using the ad hoc approach and World Health Organization (WHO) global introspection method, in short WHO method, cases 2-5 (WHO method), case 6 (WHO method, combined with the liver specific CIOMS scale), and cases 7 and 8 (Karch and Lasagna method).

Table 3 Criteria of a positive reexposure test in herb induced liver injury cases

Test result	ALTb	ALTr
Positive	< 5 N	⩾ 2 ALTb
Negative	< 5 N	< 2 ALTb
Negative	⩾ 5 N	⩾ 2 ALTb
Negative	⩾ 5 N	< 2 ALTb
Negative	⩾ 5 N	N/A
Uninterpretable	< 5 N	N/A
Uninterpretable	N/A	N/A

Details and criteria for a positive reexposure test are based on the conclusions of International Consensus Meetings. Accordingly, required data are the alanine aminotransferase (ALT) levels just before reexposure, designated as baseline ALT or ALTb, and the ALT levels during reexposure, designated as ALTr. Response to reexposure is positive, if both criteria are met: first, ALTb is < 5 N with N as the upper limit of normal, and second ALTr ⩾ 2 ALTb. Other variations lead to negative or uninterpretable results. Criteria are based on ALT values and thereby applicable to the hepatocellular type of liver injury. N/A: Not available.

Causality for Herbalife was reevaluated using the updated CIOMS scale for the hepatocellular type of liver injury (Table 5), and identical results were obtained with the original CIOMS scale (data not shown). Considering previous information on assumed hepatotoxicity by Herbalife, all eight cases were credited uniformly with +1 point to simplify assessment. The overall scores ranged from +7 to -2 points, representing a broad spectrum of causality gradings. Causality levels for Herbalife were probable (case 1), unlikely (cases 2, 3, 4 and 7), and excluded (cases 5, 6 and 8).

For most cases, the scores were low (Table 5). In 7/8 cases, the latency period until symptoms or increased liver values appeared > 90 d, resulting in only +1 point rather the +2 points usually given in other HILI or DILI cases. ALT dechallenge often was poorly documented without actual values at day 8 and around day 30, resulting in 0 points. Comedication was reported in 6/8 cases, deducting 2 points in five cases. For exclusion of non-Herbalife causes, data quality was poor and resulted in +1 point in four cases and negative points in the remaining cases. Considering previous information on Herbalife hepatotoxicity, all eight cases were uniformly credited with +1 point, since no attempt was made in any of the

Table 4 Analysis of positive reexposure tests in cases with suspected Herbalife hepatotoxicity

Cases with initially suggested positive reexposure tests
Case 1
The 63-yr old woman used a Herbalife product and experienced a positive reexposure test that was fairly well documented, but duration of product reuse was insufficiently communicated[1]. Upon first challenge, ALT was 1897 U/L and declined to 35 U/L after product discontinuation. Rechallenge increased ALT 758 U/L. Since ALTb is < 5 N and ALTr ⩾ 2 ALTb, this ascertains the positive reexposure test
Case 2
The 55-yr old woman consumed Herbalife products. Liver disease by not further specified liver values as well as a positive reexposure test was described[2]. Individual ALT values were not presented, hence data required for criteria of ALTb < 5 N and ALTr ⩾ 2 ALTb are not available. The data are uninterpretable regarding the claimed positive reexposure test
Case 3
The 48-yr old woman was on Herbalife products, when hepatocellular hepatitis was diagnosed associated with a positive reexposure test[2]. Lack of any specific ALT values prevented establishing criteria of ALTb < 5 N and ALTr ⩾ 2 ALTb. The case is uninterpretable with respect to the reexposure test
Case 4
The 78-yr old woman used Herbalife products and was diagnosed with hepatocellular liver injury based on liver values[2]. A positive reexposure test was described, but details of the test and individual ALT values were not provided. Therefore, criteria of ALTb < 5 N and ALTr ⩾ 2 ALTb cannot be ascertained. The case is uninterpretable due to lacking test criteria.
Case 5
The 30-yr old man consumed Herbalife products and experienced a biopsy proven liver disease[3]. A positive reexposure test was described, but details were not provided. An initial ALT value was reported with lack of ALT data in the further course including the reexposure test, preventing the confirmation of the essential criteria ALTb < 5 N and ALTr ⩾ 2 ALTb. Lack of these criteria leads to uninterpretable data of the test
Case 6
The 44-yr old woman used Herbalife products, experienced jaundice with increased ALT 2637 U/L[4]. Following product cessation, normalization of liver values reported, but actual ALT values were not presented. After Herbalife reuse, rise of liver values was communicated, but no details of actual ALT values given. ALTb is probably < 5 N, but ALTr is unknown. Currently, this case is uninterpretable regarding the reexposure test
Case 7
The 39-yr old woman was on Herbalife products and experienced a hepatitis, which improved after product cessation, but actual ALT values before reexposure are not communicated[5]. Recurrent increase of ALT was reported, but actual values not presented. Since ALTb and ALTr are unknown, the reexposure test is uninterpretable
Case 8
The 49-yr old woman used Herbalife products and experienced an ALT of 922 U/L, which dropped after product cessation to 793 U/L and rose to 1500 U/L after reintroduction[5]. ALTb is ⩾ 5 N and ALTr < 2 ALTb, the test is negative
Cases with initially questionable positive reexposure tests
The 60-yr old man was reported with use of Herbalife products, a histology proven liver disease, and a questionable positive rechallenge[3]. When an increase of liver values was again observed, the patient denied Herbalife consumption. Thus, no evidence for a positive reexposure test exists
The 41-yr old woman was on a Herbalife product and suffered from fulminant hepatic failure requiring liver transplantation[3]. A questionable positive reexposure test with slightly elevated liver enzymes lacking actual ALT values was described for the transplanted liver one year after transplantation, when the patient was vague about Herbalife use. Therefore, clear evidence for a positive reexposure test is missing

The eight cases correspond to those presented in Table 1, and the data of the two cases with initially questionable positive reexposure tests are derived from the literature. Required data are alanine aminotransferase (ALT) levels at baseline before reexposure, designed ALTb, and ALT levels during reexposure, designed ALTr. Response to reexposure is positive, when ALTb < 5 N and ALTr ⩾ 2 ALTb. Criteria are applicable for the hepatocellular type if liver injury. N: Upper limit of normal.

individual published cases to differentiate whether one of the used Herbalife products had been considered as potentially hepatotoxic before. Unintentional Herbalife readministration with a positive and validated reexposure result provided +3 points in one patient and no point in the remaining seven patients due to a negative reexposure test result or uninterpretable data.

DISCUSSION

Reports of positive unintentional reexposure tests in eight cases of assumed hepatotoxicity by Herbalife products initially led to a high suspicion level of liver injury for these dietary supplements; however, specific criteria for the reexposure tests and liver specific causality assessment methods were not applied[1-5]. Using specific and established criteria for reexposure tests (Table 3)[16,31], reexposure results in the study group were positive in one patient, negative in another patient, and uninterpretable in six patients (Table 4). Subsequent liver specific causality assessments using the CIOMS scale showed much lower causality levels than published before; they now were probable (n = 1), unlikely (n = 4), or even excluded (n = 3) (Tables 1 and 5). For evaluating future cases with hepatotoxicity upon reexposure, the combined use of specific criteria for reexposure tests and liver specific causality assessment methods such as the CIOMS scale are the preferred tools to achieve valid results.

Generally accepted hepatotoxicity biomarkers for all cases are lacking; when available, a positive unintentional reexposure test is still considered as a gold standard to establish causality in DILI and HILI cases[16,32-35]. Retrospective assessment of unintentional reexposure tests is cumbersome, because clinical conditions are variable, as shown in the present report (Tables 1 and 4)[1-5] and in previous case analyses[14,36-51]. Specific criteria for reexposure tests are available since 1988 (Table 3)[31] and have been incorporated in the CIOMS scale (Table 4)[16,32,34] following successful use for validation purposes[33]. For the eight cases of assumed Herbalife associated hepato-

Table 5 Causality assessment of all eight patients with primarily suspected Herbalife hepatotoxicity and an initially assumed positive reexposure test

Items for hepatocellular type of injury	Score	1	2	3	4	5	6	7	8
1 Time to onset from the beginning of Herbalife									
5-90 d (rechallenge: 1-15 d)	+2							+2	
< 5 or > 90 d (rechallenge: > 15 d)	+1	+1	+1	+1	+1	+1	+1		+1
Alternative: Time to onset from cessation of Herbalife									
≤ 15 d (except for slowly metabolized chemicals: > 15 d)	+1								
2 Course of ALT after cessation of Herbalife									
Percentage difference between ALT peak and N									
Decrease ≥ 50% within 8 d	+3								
Decrease ≥ 50% within 30 d	+2	+2							
No information	0		0	0	0	0	0	0	0
Decrease ≥ 50% after the 30th d	0								
Decrease < 50% after the 30th d or recurrent increase	-2								
3 Risk factors									
Alcohol use (drinks/d: > 2 for woman, > 3 for men)	+1								
Alcohol use (drinks/d: ≤ 2 for woman, ≤ 3 for men)	0	0	0	0	0	0	0	0	0
Age ≥ 55 yr	+1	+1	+1		+1				
Age < 55 yr	0			0		0	0	0	0
4 Concomitant drug(s)									
None or no information	0					0		0	
Concomitant drug with incompatible time to onset	0								0
Concomitant drug with compatible or suggestive time to onset	-1								
Concomitant drug known as hepatotoxin and with compatible or suggestive time to onset	-2	-2	-2	-2	-2		-2		
Concomitant drug with evidence for is role in this case (positive rechallenge or validated test)	-3								
5 Search for non Herbalife causes									
Group Ⅰ (6 causes)									
Anti-HAV-IgM		-	-	-	-	-			
Anti-HBc-IgM/HBV-DNA		-	-	-	-	-			
Anti-HCV/HCV-RNA		-	-	-	-	-			
Hepato-biliary sonography/colour Doppler sonography of liver vessels/endosonography/CT /MRC		+	-	-	-	-			
Alcoholism (AST/ALT ≥ 2)		-	-	-	-	-		-	-
Acute recent hypotension history (particularly if underlying heart disease)		-	-	-	-	-			-
Group Ⅱ (6 causes)									
Complications of underlying disease(s), such as sepsis, autoimmune hepatitis, chronic hepatitis B or C, primary biliary cirrhosis or sclerosing cholangitis, genetic liver diseases		-	-	-	-				-
Infection suggested by PCR and titer change for									
CMV (Anti-CMV-IgM/IgG)		-	-	-	-				
EBV (Anti-EBV-IgM/IgG)		-	-	-	-				
HEV (Anti-HEV-IgM/IgG)						+			
HSV (Anti-HSV-IgM/IgG)									
VZV (Anti-VZV-IgM/IgG)									
Evaluation of group Ⅰ and Ⅱ									
All causes - group Ⅰ and Ⅱ - reasonably ruled out	+2								
The 6 causes of group Ⅰ ruled out	+1	+1	+1	+1	+1				
5 or 4 causes of group Ⅰ ruled out	0								
Less than 4 causes of group Ⅰ ruled out	-2						-2	-2	-2
Non Herbalife cause highly probable	-3					-3			
6 Previous information on hepatotoxicity of Herbalife									
Reaction labelled in the product characteristics	+2								
Reaction published but unlabelled	+1	+1	+1	+1	+1	+1	+1	+1	+1
Reaction unknown	0								
7 Response to readministration									
Doubling of ALT with Herbalife alone, provided ALT below 5 N before reexposure	+3	+3							
Doubling of ALT with Herbalife and herb(s) or drug(s) already given at the time of first reaction	+1								
Increase of ALT but less than N in the same conditions as for the first administration									
Other situations	0		0	0	0	0	0	0	0
Total points for patients		+07	+02	+01	+02	-01	-02	+01	0

In all eight patients with initially suspected Herbalife hepatotoxicity (Tables 1 and 4), causality assessment for Herbalife was performed with the updated CIOMS scale for the hepatocellular type of liver injury. The symbol "-" denotes that the obtained result was negative and that of "+" was positive, whereas lack of a symbol indicates missing data. Regarding risk factor of alcohol use, 1 drink commonly contains about 10 g ethanol. Total points provide causality levels: ≤ 0, excluded; 1-2, unlikely; 3-5, possible; 6-8, probable; ≥ 9, highly probable. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CIOMS: Council for International Organizations of Medical Sciences; CMV: Cytomegalovirus; CT: Computer tomography; EBV: Epstein Barr virus; HAV: Hepatitis A virus; HBc: Hepatitis B core; HBsAg: Hepatitis B antigen; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HEV: Hepatitis E virus; HILI: Herb induced liver injury; HSV: Herpes simplex virus; MRC: Magnetic resonance cholangiography; N: Upper limit of normal; VZV: Varicella zoster virus.

toxicity, no information was available whether specific criteria were used to assess the reexposure result as positive (Table 4)[1-5].

Notably, intentional reexposure tests are obsolete due to high risks to the health of the patients. In the past, this kind of approach provided validated test results, since appropriate test conditions could be established prospectively, facilitating data evaluation. For decades, however, only unintentional reexposure test results with scattered data are available as evidenced in the present Herbalife study[1-5], allowing retrospective rather than prospective evaluation (Table 4). These data gaps influence the CIOMS scoring, with only the one patient receiving +3 points indicating a positive result (case 1), whereas all other patients scored 0 points for the reexposure item (cases 2-8) (Table 5). This low score is even more remarkable since the CIOMS scale will award +2 points for an appropriate rechallenge time to onset of 1-15 d, and +1 point when the time to onset is > 15 d[32,34]. In future case reports of hepatotoxicity, therefore, special care should be provided to appropriate use of accepted criteria for reexposure tests.

Data problems of reexposure cases are not confined to Herbalife products (Table 4) but represent a general problem extending to liver injury by all herbal drugs, dietary supplements and herbal products[14,36-51]. Analysis of 30 cases within the last three decades claiming a positive reexposure test revealed that in many cases detailed descriptions of the reexposure test and actual ALT values were lacking. This was most evident in short case reports, often presented as a letter to the editor, and in case series. In retrospect, a positive reexposure test has been confirmed in only 13/30 cases (43%)[16], as ascertained by established criteria published previously[31]. Of note, none of these reports communicated criteria for the evaluation of the observed reexposure test[14,16,36-51].

The use of inappropriate causality assessment methods in the analyzed case reports is difficult to reconcile[1-5]. An ad hoc approach was applied in one patient (case 1)[1], with reassessment[3] by the WHO method[52]. This method was also used in four other patients (cases 2-5) alone[2,3] or in one patient (case 6) combined with the CIOMS scale[4]. In the remaining patients (cases 7 and 8)[5], assessment was achieved with the Karch and Lasagna method[53]. None of these approaches except the CIOMS scale is liver specific; the methods are not validated for hepatotoxicity and obsolete under these conditions. Clear preference should have been given to the CIOMS scale, with all its strengths and weaknesses[16,31-35,54]. The CIOMS scale considers all core elements of hepatotoxicity (Table 5)[34]; it was developed by an international expert panel and validated by cases with positive reexposure tests as gold standard[32,33]. CIOMS based assessment has shown good sensitivity (86%), specificity (89%), and positive predictive value (93%) and negative predictive value (78%)[33].

Surprisingly, the WHO method[52] used in most of the analyzed studies[1-5] has not been validated for any adverse drug reaction[55,56], its global introspection by experts has been shown to be neither reproducible nor valid[57]; it is not reference validated or quantitative[52,54-61], and reliability, sensitivity, specificity, positive and negative predictive values are unknown[52,54-56,61]. Both the questions and the possible answers posed to the assessor are ambiguous[54,56]. Specifically, the assessor considers factors that might causally link one or more drugs to an observed adverse drug reaction (ADR), lists all factors, weighs their importance, and decides the probability of drug causation[57]; but no checklist is given or level of strength required. Its scope is also limited since it cannot discriminate between a positive and a negative correlation, thereby stimulating overdiagnosing and overreporting[52]. The WHO method ignores data uncertainties, *e.g.*, in daily dose, temporal association, start, duration, and end of herbal use, time to onset of the ADR, and course of liver values after herb discontinuation. Insufficiently considered or ignored are comedications, preexisting liver diseases, numerous alternative explanations, and exclusion of virus infections by hepatitis A-C, CMV, EBV, HSV, and VZV[56,59,60].

Also for case evaluation[5] by the old Karch and Lasagna method[53], subjective judgement is needed for many steps, making the method more prone to bias[35]. Though commonly applied by the Spanish Pharmacovigilance Centres[5], this method is not used by the Spanish Group for the Study of Drug-induced Liver Disease[14,35,62,63]. For unknown reasons, this group did not tabulate any of the suspected Spanish Herbalife cases together with HILI cases that had been assessed by the CIOMS scale[14].

Assessment of the suspected Herbalife cases revealed various shortcomings and possible confounders creating concern in the present study (Tables 1, 2, 4 and 5). This is a general problem in retrospective analyses[1-5], case collection from nationwide hospitals[2,3], and spontaneous reports derived from regulatory agencies[5], as are challenges of causality assessments in HILI cases[16,30,55,56,58-61,64-68]. In a recent comprehensive review article of herbal and dietary supplement hepatotoxicity, careful analysis included the use of the CIOMS scale, being the diagnostic tool of choice in the literature pertaining to herbal hepatotoxicity[68]. This is supported by an actual evaluation of 573 HILI cases, which showed that the CIOMS scale was applied in 275/573 cases (48%)[30]. Possible or likely alternative diagnoses were evident in 278/573 cases (48.5%) of suspected HILI cases; causality assessment was impeded in 165/573 patients (29.0%), resulting in diagnostic problems in 77.5% of all cases[30]. Given these limitations, actual discussions of suspected Herbalife hepatotoxicity are understandable regarding case data quality and the preferred tool to assess causality[69,70], issues also recognized before[16,30,34] and in the present study (Tables 1, 2, 4 and 5). In reference to three case series of suspected Herbalife hepatotoxicity from Israel[2], Switzerland[3], and Spain[5], the opinion has been expressed that these series have utilized generally accepted causality assessment for herbal hepatotoxicity[70]. In these three case series, causality assessment methods were the WHO method[52] in two series[2,3] and

the Karch and Lasagna method[53] in one series[5]. All these approaches are liver unspecific, not validated for hepatotoxicity cases, and therefore inappropriate tools assessing causality in HILI cases[16,30,56,59-61]. The National Institutes of Health LiverTox specifically addressed the item of causality in hepatotoxicity cases and focused primarily on using the CIOMS scale, whereas the WHO method and the Karch and Lasagna method were not discussed and not even mentioned, thereby simply ignored[65,66], as in a careful review article published recently[68].

Incomplete data of viral serology in the present study (Tables 1, 2, and 5) is an issue also for DILI cases[71]. It may be of relevance for HEV infection, which is poorly tested but confirmed in one patient (Table 2) and easily overseen, as demonstrated in recent reports[72,73]. Carefully conducted studies have shown that 21% of patients with criterion-referenced DILI did not have DILI at all, but had HEV infection[72]. Similarly, among 318 patients with suspected DILI, 50 (16%) were tested positive for anti HEV IgG and nine (3%) for anti HEV IgM[73]. Moreover, 22% of patients with autochthonous hepatitis E were erroneously thought to have criterion-referenced DILI[72]. The authors comment and believe that these findings are likely to be applicable to other studies in the developed world and emphasize that DILI cannot securely be diagnosed without HEV testing and exclusion. This certainly also applies to suspected HILI cases.

In conclusion, the analysis of cases of initially assumed Herbalife hepatotoxicity with positive reexposure tests and high causality levels revealed both lacking criteria for the tests and missing use of a liver specific causality assessment method. Based on these shortcomings, causality levels for Herbalife had to be downgraded. Future assessment of liver injury by dietary supplements will require thorough evaluation of both unintentional reexposure tests by specific and established criteria and causality by liver specific methods.

COMMENTS

Background

Considerable interest focused on the question whether few Herbalife products are potentially hepatotoxic, but overall data quality of reported cases was mixed due to confounding variables and missing criteria for the firm exclusion of alternative explanation and/or a well-based causality attribution. For hepatotoxicity cases, stringent causality assessment is mandatory, since the culprit remains undetected in up to 38% of severe liver disease. Alternative causes are frequently found, with up to 47% in initially assumed drug induced liver disease, and with an average of 49% in initially suspected herb induced liver injury.

Research frontiers

A positive reexposure test is commonly considered as gold standard to establish causality for hepatotoxicity by drugs and herbs, but in published reports, test conditions and results rarely are presented with specific details. Therefore, in cases with assumed hepatotoxicity and a positive unintentional reexposure test, the question should be answered whether specific and well established reexposure criteria were fulfilled.

Innovations and breakthroughs

This is the first study that critically analyzes reported positive unintentional reexposure tests in initially suspected liver injury by herbal dietary supplements, using published criteria of the test.

Applications

The data can contribute to a more sophisticated and critical approach assessing results of an unintentional reexposure retrospectively, taking into account established test criteria.

Terminology

Positive reexposure test: Though commonly claimed as a gold standard to establish the diagnosis of liver injury by drugs and herbs, published reports usually lack any definition. For a positive reexposure test, a baseline value alanine aminotransferase (ALT) below 5 upper limit of normal (N) before reexposure is required, with N as the upper limit of the normal value, and a doubling of the ALT value at reexposure as compared to the ALT value at baseline. Reexposure tests are unintentional and require retrospective analysis of mostly scattered data. Though previously providing good results due to prospective assessment, intentional reexposure tests are obsolete to due high risks.

Peer review

The authors analyze the reported eight cases of assumed Herbalife hepatotoxicity with a positive unintentional reexposure test in this well conducted study. Various dietary supplements may cause liver injury. Therefore, it is interesting for determining whether there is a clear causality between some Herbalife products and hepatotoxicity. The analytical approaches are described in detail, the results are impressive.

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BRIEF ARTICLE

Comparative effectiveness of traditional chemoembolization with or without sorafenib for hepatocellular carcinoma

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Abstract

AIM: To compare the overall survival (OS) and progression-free survival (PFS) with associated adverse events (AE) in patients with unresectable hepatocellular carcinoma (HCC) treated with transarterial chemoembolization (TACE) + sorafenib *vs* TACE alone.

METHODS: In this retrospective cohort study we collected data on all consecutive patients with a diagnosis of unresectable HCC between 2007 and 2011 who had been treated with TACE + sorafenib or TACE alone. We hypothesized that the combination therapy is superior to TACE alone in improving the survival in these patients. Data extracted included patient's demographics, etiology of liver disease, histology of HCC, stage of liver disease with respect to model of end stage liver disease score and Child-Turcotte-Pugh (CTP) classification and Barcelona Clinic Liver Cancer (BCLC) staging for HCC. Computed tomography scan findings, alpha fetoprotein levels, number of treatments and related AE were also recorded and analyzed.

RESULTS: Of the 43 patients who met inclusion criteria, 13 were treated with TACE + sorafenib and 30 with TACE alone. There was no significant difference in median survival: 20.6 mo (95%CI: 13.4-38.4) for the TACE + sorafenib and 18.3 mo (95%CI: 11.8-32.9) for the TACE alone (P = 0.72). There were also no statistically significant differences between groups in OS (HR = 0.82, 95%CI: 0.38-1.77; P = 0.61), PFS (HR = 0.93, 95%CI: 0.45-1.89; P = 0.83), and treatment-related toxicities (P = 0.554). CTP classification and BCLC staging for HCC were statistically significant (P = 0.001, P = 0.04 respectively) in predicting the survival in patients with HCC. The common AE observed were abdominal pain, nausea, vomiting and mild elevation of liver enzymes.

CONCLUSION: Combination therapy with TACE + sorafenib is safe and equally effective as TACE alone in patients with unresectable HCC. CTP classification and BCLC staging were the significant predictors of survival. Future trials with large number of patients are needed to further validate this observation.

Key words: Hepatocellular carcinoma; Transarterial chemoembolization; Sorafenib; Survival; Adverse events

Core tip: The incidence of hepatocellular carcinoma (HCC) is increasing and there is a need for better treatment modalities. Transarterial chemoembolization (TACE) and sorafenib are the main course of treatment for unresectable HCC. However there is an emphasis to combine them to improve survival. There is very

limited data available to compare the effectiveness of TACE alone *vs* combination with sorafenib. Our results showed equal efficacy for both treatment arms without compromising adverse events. Child-Turcotte-Pugh classification and Barcelona Clinic Liver Cancer staging were significant predictors of survival. This study is the first reported in the literature comparing the outcome when treated with TACE alone *vs* TACE + sorafenib in United States patients.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of death from cancer worldwide and ninth leading cause of death from cancer in United States. It accounts for over 12000 deaths per year in the United States. The incidence of HCC is increasing dramatically primarily due to the aging of people infected with the hepatitis C virus (HCV)[1]. During the past two decades, the incidence of HCC in the United States has tripled while the 5-year survival rate for patients who do not have a liver transplant (LT) remains < 12%. The 5-year cumulative risk for the development of HCC in patients with cirrhosis ranges from 5% to 30%, with the highest risk in patients infected with HCV and has decompensated disease[2].

There are several potentially curative or palliative approaches to the treatment of HCC. The choice of treatment is driven by the degree of hepatic dysfunction as calculated by Child-Turcotte-Pugh (CTP) classification (Table 1), cancer stage as per Barcelona Clinic Liver Cancer (BCLC) staging System for HCC (Table 2) and the resources available. When the lesion is small, the patient may be a surgical candidate if there is preservation of liver function. However < 5% of patients are deemed resectable with the acceptable risk[3]. Patients who meet the Milan criteria (1 lesion ≤ 5 cm or 3 lesions ≤ 3 cm each with no vascular invasion) may be listed for LT[4]. However, even with the priority status afforded by the model of end stage liver disease (MELD) system, the wait may be prolonged and complications may include tumor growth[5,6]. Patients with no or compensated cirrhosis and no vascular invasion but with large or multifocal lesions are considered to have intermediate-stage HCC. In these patients, if LT is not possible, local ablative therapy is the next best option[7].

Loco-regional treatment with transarterial chemoembolization (TACE) is offered to patients awaiting LT or as a palliative therapy to those who do not meet the Milan criteria for LT[8-12]. Treatment with repeated TACE shows significant survival benefits in patients with metastatic HCC who have preserved liver function[13]. A meta-analysis of randomized, controlled trials assessing the use of TACE as primary palliative treatment for HCC showed that it was associated with a 20%-25% improvement in 2-year survival rate *vs* conservative treatment[14]. The limitation of TACE is the incomplete target lesion necrosis, which requires repeated treatments in many patients. Despite the efficacy in local disease control and symptomatic relief, long-term survival rates in HCC patients after TACE remain low due to local and/or regional recurrence, as well as distant metastasis[15]. Effective systemic chemotherapy for advanced HCC is also needed to improve the overall survival of these patients[16].

Sorafenib, an orally active multikinase inhibitor with effects on tumor-cell proliferation and tumor angiogenesis, was initially identified as a Raf kinase inhibitor that acts by inhibiting the serine-threonine kinase Raf-1 and B-Raf. It also inhibits vascular endothelial growth factor receptors 1, 2 and 3; platelet-derived growth factor receptor β; and receptor tyrosine kinase receptor tyrosine kinases[17]. In a recent randomized, controlled trial (Sorafenib HCC Assessment Randomized Protocol also known as SHARP), patients with advanced HCC who were treated with sorafenib *vs* placebo had a 37% increase in survival (equivalent to a gain of 2 to 3 mo of life)[18,19]. Another meta-analysis of randomized controlled trials showed that survival rates were higher in patients treated with sorafenib-based *vs* placebo-based chemotherapy[20].

Since TACE is the most widely used primary treatment of HCC before LT or as a palliative therapy (in patients who are not LT candidates), and sorafenib is the only proven effective systemic treatment for advanced HCC[21], there is a strong rational to combine both treatment modalities[22]. Combining TACE with agents with anti-angiogenic properties is a promising strategy because TACE is thought to cause local hypoxia, resulting in a temporary increase in levels of VEGF, and sorafenib provides anti-angiogenesis activity by inhibiting VEGF levels. In a recent study, plasma VEGF decreased from 93 to 67 ng/L in patients treated with sorafenib + TACE[23].

Results from a large phase Ⅱ randomized, double-blind, placebo-controlled trial (SPACE study) showed that the concurrent administration of TACE and sorafenib (TACE + sorafenib) has a manageable safety profile and suggested that time to progression and time to vascular invasion or extra-hepatic spread may be improved *vs* treatment with TACE alone[24]. Another study by Pinter *et al*[25] showed no difference in survival in patients with advanced stage HCC treated with TACE alone *vs* sorafenib alone ($P = 0.377$). However, several other studies showed improved progression-free median survival and disease control rate in patients with advanced HCC who were treated with TACE and sorafenib[26-29]. Nevertheless, very few studies have compared overall survival (OS) and progression-free survival (PFS) in patients treated with TACE *vs* combination therapy with sorafenib. Chung *et al*[28] from South Korea are conducting a phase Ⅱ study

Table 1 Child-Turcotte-Pugh scoring system and classification for patients with chronic liver disease

Measures	1 point	2 points	3 points
Serum total bilirubin (mg/dL)	⩽ 2	2-3	> 3
Serum albumin (g/dL)	> 3.5	2.8-3.5	< 2.8
INR	< 1.7	1.71-2.30	> 2.30
Ascites	None	Mild	Moderate to severe
Hepatic encephalopathy	None	Grade Ⅰ-Ⅱ (or suppressed with medication)	Grade Ⅲ-Ⅳ (or refractory)
CTP points	CTP class	One year predicted survival	
5-6	A	100%	
7-9	B	81%	
10-15	C	45%	

INR: International normalized ratio; CTP: Child-Turcotte-Pugh.

Table 2 Barcelona Clinic Liver Cancer staging system for hepatocellular carcinoma

Stage of HCC	Tumor features	Child-Pugh classification	Performance status test	Treatment
Stage 0	Single < 2 cm carcinoma *in situ*	Child-Pugh A	0	Resection
Stage A	Single < 5 cm or 3 nodules < 3 cm	Child-Pugh A-B	0	Liver transplant, percutaneous ethanol injection, radiofrequency ablation
Stage B	Single > 5 cm or multi-nodular	Child-Pugh A-B	0	TACE
Stage C	Portal vein invasion	Child-Pugh A-B	1-2	Sorafenib
Stage D	Distant metastasis	Child-Pugh A-B	3-4	Symptomatic

HCC: Hepatocellular carcinoma; TACE: Transarterial chemoembolization.

on the safety, tolerability and efficacy of TACE and sorafenib in patients with HCC (START trial). The study is currently ongoing and an interim analysis revealed that the disease control rate was 91.2% while the overall response rate was 52.4%; the authors concluded that combination therapy is safe and effective with no unexpected side effects.

A recently published retrospective observational study by Qu *et al*[30] conducted in China showed that median survival time was significantly longer in patients with HCC treated with sorafenib and TACE *vs* TACE alone (27 mo *vs* 17 mo, $P = 0.001$). Despite the positive outcomes reported with the combined therapy, a clinical trial that enrolled a small number of patients was stopped prematurely due to adverse events (AE) and safety concerns with the combination therapy of high-dose doxorubicin-based TACE regimen and sorafenib[31].

Hypothesis

Due to the limited data regarding survival in patients with HCC - particularly those in the United States - treated with these different treatment modalities, we performed a retrospective cohort study of patients with unresectable HCC who were treated with TACE alone or TACE + sorafenib. The primary aim of the study was to compare the efficacy including benefits and harms of TACE alone *vs* combination therapy with sorafenib in patients with unresectable, non-transplantable HCC. We hypothesized that the combination therapy with TACE + sorafenib is superior to TACE alone in improving the survival in patients with advanced HCC. The secondary aim of the study was to find out the significant predictors of survival in these patients.

MATERIALS AND METHODS

A retrospective cohort study was conducted at the James Haley VA hospital after IRB approval (IRB Pro 000005448). Data was collected on all consecutive patients with a diagnosis of unresectable HCC from January 1, 2007 through December 31, 2011.

Criteria

Inclusion criteria: (1) patients age above 18 with unresectable biopsy-proven HCC who were not a candidate for LT; (2) patients who had been treated with TACE alone or TACE + sorafenib; and (3) patients with Child's A and B cirrhosis.

Exclusion criteria: (1) patients with CHILD's C cirrhosis and BCLC stage D for HCC; (2) liver transplant recipients; (3) patients with prior liver resection for HCC; and (4) patients who did not receive TACE as primary therapy.

Outcome measures

The primary outcome was OS and mortality. The secondary outcomes were PFS (where progression was defined as an increase in tumor size and MELD score), and AE associated with two treatments modalities. Treatment-related AE were assessed using the Common Terminology Criteria for AE (CTCAE) version 4.0.

Data abstraction

Data extracted included patient's demographics, etiology of liver disease, histology of HCC, stage of liver disease with respect to MELD score, 3.78 [Ln serum bilirubin (mg/dL)] + 11.2 (Ln INR) + 9.57 [Ln serum creatinine (mg/dL)] + 6.43, CTP classification and BCLC staging for HCC[32]. CT scan findings (pre and post treatment), alpha fetoprotein (AFP) levels during the treatment, number of TACE or TACE + sorafenib treatments, and treatment AE were also recorded. Data on patient status (alive *vs* deceased *vs* progression) was collected periodically until the last follow-up which was November 30th, 2012.

Description of treatments

TACE: Hepatic artery obstruction was performed during

Table 3 Comparison of demographic and disease characteristics of patients with hepatocellular carcinoma in both treatment groups

Characteristic	TACE + sorafenib (n = 13)	TACE alone (n = 30)	P value
Age (yr)	61.4 ± 7.5	59.2 ± 7.4	0.39
Etiology			0.18
Alcohol	2 (15.4)	1 (3.4)	
Hepatitis C	6 (46.1)	17 (56.6)	
Hepatitis C and alcohol	3 (23.1)	11 (36.6)	
Non-alcohol/non-hepatitis C	2 (15.4)	1 (3.4)	
HCC histology			0.86
Poorly differentiated	1 (7.6)	3 (10.0)	
Moderately differentiated	7 (53.8)	13 (43.3)	
Well differentiated	5 (38.6)	14 (46.7)	
CTP classification			0.69
A	11	23	
B	2	7	
BCLC staging for HCC			0.004
A	6	22	
B	2	8	
C	5	0	
BCLC staging for HCC (excluding stage C)			0.98
A	6	22	
B	2	8	
MELD score	8.8 ± 2.3	9.8 ± 2.9	0.29
AFP (ng/mL)	6.6 (2.3-745)	8.1 (1.9-6000)	0.96
Tumor size seen on CT with the largest diameter (cm)	4 (1.5 -16.7)	3.1 (1.4 -5.8)	0.58

Data are summarized as the mean ± SD or median (range). HCC: Hepatocellular carcinoma; TACE: Transarterial chemoembolization; CTP: Child-Turcotte-Pugh; BCLC: Barcelona Clinic Liver Cancer; MELD: Model of end stage liver disease; AFP: Alpha fetoprotein; CT: Computed tomography.

an angiographic procedure and is combined with the injection into the hepatic artery of chemotherapeutic agents, mixed with lipiodol. Hepatic angiography was initially done to identify all arteries feeding the tumor by intervention radiologist. After the tumoral arterial supply was assessed, the catheter was introduced into the target artery. The catheter was then advanced to interrupt the blood flow as close to tumor as possible to minimize necrosis of the surrounding area. Particulate used for TACE was drug eluting microspheres (LC beads) about 300-500 micron in size. Chemotherapeutic agents (Doxorubicin 75-150 mg with a mean of 125 mg as per treating physician's discretion) were adsorbed on the particulate bead and then injected into the tumor through the microcatheter.

Sorafenib: An oral starting dose of 200 mg twice daily was initiated by the treating oncologist and increased to 400 mg twice daily in the majority of patients. The decision to continue or stop the treatment because of AE was made by the oncologist.

Statistical analysis

OS was calculated from the first day of initial treatment with TACE or TACE + sorafenib to status at last contact (dead *vs* alive). PFS was calculated similarly except that we noted the progression as either increase in tumor size and MELD score or death at last contact. Treatment outcomes of the TACE *vs* TACE + sorafenib groups were compared. Time-to-event data analysis was estimated by the Kaplan-Meier survival method and compared by the Log-rank test. Differences in treatment effect between the TACE and TACE + sorafenib groups, including OS and PFS, were also assessed using the Cox proportional hazard model and summarized as HR along with 95%CI. All statistical tests were two-sided, and a P value $\leqslant 0.05$ was considered statistically significant. Differences in treatment effects for dichotomous outcomes were compared using Fisher exact test. All the analyses were done using STATA statistical analysis[33,34].

RESULTS

Patients and treatment characteristics

Forty-three consecutive patients were eligible for inclusion. All patients were male and underwent liver biopsy prior to treatment to confirm the diagnosis of HCC. At diagnosis, the MELD score ranged from 6 to 22 (mean 9.5). There were no statistically significant differences in any of the baseline characteristics (age, etiology of liver disease, histology of HCC, CTP classification, MELD score, AFP, tumor size) between the two treatment groups with the exception of the BCLC stage C (Table 3). The maximum number of TACE sessions per patient was 6 with an average of 1.9 sessions per patient. The average time from the first TACE treatment to the initiation of sorafenib was 8 mo. The mean duration of sorafenib treatment was 11.7 mo (range 1.9-42 mo), and the mean follow-up duration was 23 mo (range 3-56 mo). None of the patients were lost to follow up and all the clinical encounters were completed and recorded.

Outcomes

OS and PFS: Thirty patients (70%) were treated with TACE alone and 13 (30%) with combination therapy (TACE + sorafenib). Overall HCC-related mortality was 74% (32/43 patients). Of the 32 patients who died, 23 (72%) received TACE alone and 9 (28%) received combination therapy (P = 0.70). There was no significant difference in median survival time between groups: 20.6 mo (95%CI: 13.4-38.4) for the TACE + sorafenib group and 18.3 mo (95%CI: 11.8-32.9) for the TACE group (P = 0.72). There was no statistically significant difference in OS between the 2 treatment groups (Figure 1A). The HR for OS was 0.82 (95%CI: 0.38-1.77), which indicated an 18% hazard reduction in mortality with TACE + sorafenib *vs* TACE, however, this difference was not statistically significant (P = 0.61). The HR for PFS was also not statistically significant 0.93 (95%CI: 0.45-1.89; P = 0.83) (Figure 1B).

The HR for OS was 0.56 (95%CI: 0.21-1.47; P = 0.24) and for PFS was 0.70 (95%CI: 0.3-1.6; P = 0.41) after excluding BCLC stage C patients. There was a decrease in hazard in favor of the combination therapy (after excluding BCLC stage C patients), but the difference was not

Table 4 Statistical analysis for each covariate in univariate cox regression model predicting overall survival

Characteristic	HR (95%CI)	*P* value
Age (yr)	0.99 (0.95-1.04)	0.79
Etiology	1.04 (0.66-1.63)	0.86
Alcohol		
Hepatitis C		
Hepatitis C and alcohol		
Non-alcohol/non-hepatitis C		
HCC histology	0.73 (0.43-1.25)	0.26
Poorly differentiated		
Moderately differentiated		
Well differentiated		
CTP classification	3.84 (1.74-8.51)	0.001
A		
B		
BCLC staging for HCC	1.58 (1.02-2.46)	0.04
A		
B		
C		
BCLC staging for HCC (excluding stage C)	1.5 (0.66-3.43)	0.34
A		
B		
MELD score	1.1 (0.98-1.23)	0.08
AFP (ng/mL)	0.99 (0.82-1.20)	0.98
Tumor size seen on CT with the largest diameter (cm)	1.06 (0.93-1.20)	0.41

HCC: Hepatocellular carcinoma; CTP: Child-Turcotte-Pugh; BCLC: Barcelona Clinic Liver Cancer; MELD: Model of end stage liver disease; AFP: Alpha fetoprotein; CT: Computed tomography.

Table 5 Adverse events attributed to both treatment arms

Adverse events (CTCAE grades 1-3)	TACE alone	TACE + sorafenib	*P* value
Hand foot skin reaction	0	2	0.554
Diarrhea	0	1	
Hypertension (mild)	0	1	
Abdominal pain (mild)	6	1	
Nausea, vomiting	3	0	
Elevated liver enzymes (< 2 times of normal limits)	1	2	

TACE: Transarterial chemoembolization; CTCAE: Common terminology criteria for adverse events.

statistically significant.

AFP levels ranged from 1.86 to 6000 (mean 85.9). Four patients had AFP levels above 400 and one patient with AFP of 6000 in TACE alone group (BCLC stage B with no vascular invasion). However there was no statistically significant difference of OS with HR of 0.81 (95%CI: 0.36-1.86; $P = 0.63$) and PFS with HR of 0.93 (95%CI: 0.44-1.97; $P = 0.85$), after removing these patients.

CTP classification of severity of liver disease (class B) and BCLC staging for HCC (stage C) were statistically significant ($P = 0.001$, $P = 0.04$ respectively) when analyzed by univariate Cox regression model in predicting the outcome and OS in patients with HCC (Table 4). This observation emphasizes the fact that patients with advance liver disease and higher stage of HCC have the worst outcome. Age, etiology of liver disease, tumor size and histology, MELD score and AFP level did not impact the OS in our cohort of patients.

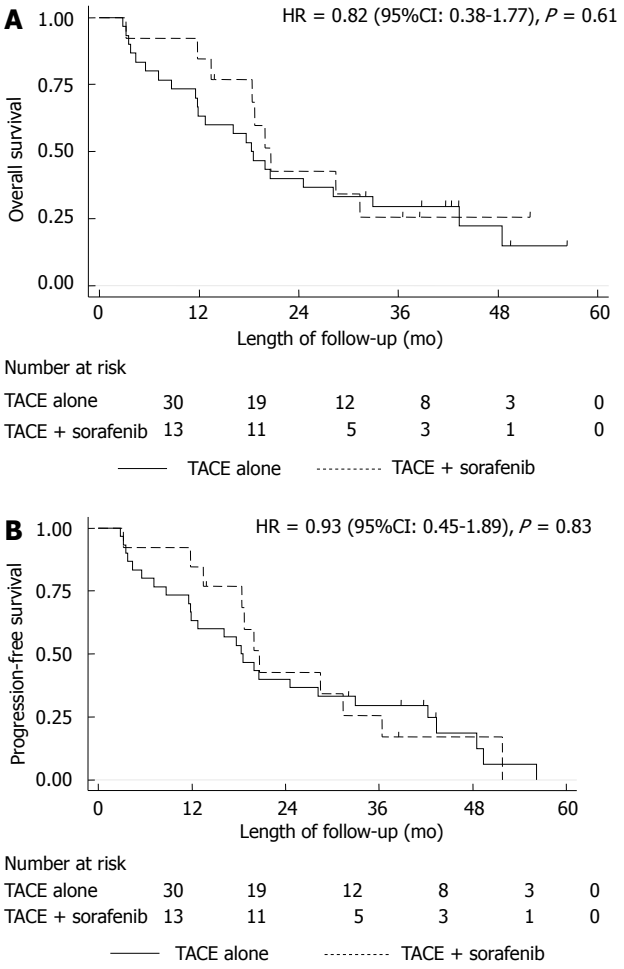

Figure 1 Kaplan-Meier survival. A: Kaplan-Meier overall survival for transarterial chemoembolization (TACE) alone and TACE + sorafenib; B: Kaplan-Meier progression-free survival for TACE alone and TACE + sorafenib.

AE: The most common AE observed in our cohort of patients were abdominal pain, nausea, vomiting and mild elevation of liver enzymes. Specifically 2 patients had hand-foot skin reaction syndrome secondary to sorafenib after increasing the dose from 200 mg twice daily to 400 mg twice daily. The treatment with sorafenib was then stopped for few weeks and then re-introduced with a lower dose (200 mg twice daily) without any side effects. None of the side effects secondary to both treatments were life-threatening. There was no statistically significant difference in the AE between the two treatment groups (Table 5). No grade 4 or above AE as per CTCAE version 4.0 were observed with either TACE or sorafenib.

DISCUSSION

To our knowledge this is the first study to compare the outcome of patients with HCC treated with two different modalities (TACE alone *vs* TACE + sorafenib) in United States patients. Survival was slightly better in

the TACE + sorafenib group than the TACE group, as demonstrated by an 18% hazard reduction in mortality, however this difference was not statistically significant most likely because of small sample size. The median survival was slightly prolonged in patients treated with TACE + sorafenib *vs* TACE alone (20.6 mo *vs* 18.3 mo). The observed effect of TACE + sorafenib compared with TACE alone was seen without any significant differences in AE. Furthermore, the patient's data was not compromised as none of the patients were lost to follow up and all the clinical encounters were completed. Both treatment groups were comparable in terms of disease processes and prognostic factors. The AEs related to treatment with TACE and sorafenib were comparable to those reported in the literature[27]. CTP classification of severity of liver disease and BCLC staging for HCC were the only significant predictors of survival in our patients when analyzed in a univariate cox regression model.

Our findings support the findings of a recently published phase Ⅲ study in which Japanese and Korean patients with advanced HCC were randomized to receive sorafenib or placebo (1:1) after TACE therapy. Median times to progression (TTP) in the sorafenib and placebo groups were 5.4 and 3.7 mo, respectively (HR = 0.87, 95%CI: 0.70-1.09; P = 0.252). The HR in sorafenib/placebo for overall survival was 1.06 (95%CI: 0.69-1.64; P = 0.790) and they concluded that combination therapy with TACE + sorafenib is not superior to TACE alone[35].

However, our findings are in contrast with those from a recent study assessing the effectiveness of TACE + sorafenib *vs* TACE in Chinese patients; Qu *et al*[30] reported a statistically significant improvement in median survival time with the combination therapy when compared to TACE alone (27 mo *vs* 17 mo, P = 0.001). The primary reason for the positive results observed in the study by Qu *et al*[30] may be attributed to their larger sample size (90 patients) in their study compared with our study with 43 patients.

Furthermore, the study by Qu *et al*[30] is limited by a relatively uneven duration of patient follow-up for the compared treatment modalities (25 mo for TACE alone and 46 mo for combination therapy). In contrast, our study has even and longer duration of follow-up (56 mo for TACE alone and 52 mo for combination therapy). Nevertheless, the results from both studies need to be confirmed in a randomized, placebo-controlled prospective study. A similar phase Ⅲ study (SPACE trial) that is currently ongoing will evaluate differences in outcome between the 2 treatment groups[24]. Three hundred and seven patients with unresectable HCC and CHILD's A cirrhosis were enrolled. Preliminary data showed statistically significant advantage of sorafenib + TACE over placebo + TACE in time to progression of HCC (TTP median 169 d, HR = 0.797, 95%CI: 0.588-1.080; P = 0.072).

Limitations of our study include its retrospective study design, the small number of patients included, and a patient population from a single institute. In addition, the decision to treat with TACE *vs* TACE + sorafenib was made by the treating physicians who might be prone to selection bias due to their belief in the superiority of one of the treatments. The benefit difference by looking at the survival curves, occurred early during the course of treatment suggesting the possibility of selection bias. There is also no data available on quality of life differences between these 2 cohorts of patients. A major strength of this study is the long duration of follow-up post-therapy (longest follow up of 56 mo) which enabled us to capture most treatment-related events and no lost to follow up. This is also the first reported study from the United States comparing the effectiveness of these two treatment modalities in patients with HCC.

In conclusion, combination therapy with TACE + sorafenib is safe and equally effective as TACE alone without any unexpected AE, in patients' with unresectable HCC. The median survival time was prolonged by 2 mo in the combination treatment group, but it was not statistically significant. CTP classification and BCLC staging for HCC were the only significant predictors of survival emphasizing the fact that patients with advance liver disease and higher stage of HCC have the worst outcome. Future trials with large number of patients are needed to further validate this observation.

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COMMENTS

Background

The incidence of hepatocellular carcinoma (HCC) is increasing especially in patients with chronic hepatitis C and there is a need for better treatment modalities to improve the overall survival. Transarterial chemoembolization (TACE) and sorafenib are the main course of treatment for unresectable HCC in patients who are not candidates for liver transplantation. However there is an emphasis to combine these two treatment modalities to improve the overall survival. To date, there is very limited data available especially in United States patients, to compare the effectiveness of TACE alone *vs* TACE + sorafenib.

Research frontiers

This study looked at the outcome of patients with unresectable HCC treated with TACE alone *vs* TACE + sorafenib. The primary outcome was to assess the overall and progression-free survival among the two groups.

Innovations and breakthroughs

This study showed equal efficacy for both treatment arms without compromising adverse events. The median survival time was prolonged by 2 mo in the combination treatment group, but it was not statistically significant. Child-Turcotte-Pugh (CTP) classification and Barcelona Clinic Liver Cancer (BCLC) staging for HCC were the only significant predictors of survival in these patients. This study is the first reported in the literature comparing the outcome of patients with HCC when treated with TACE alone *vs* TACE + sorafenib in United States patients.

Applications

Combination therapy is safe and effective in patients with unresectable HCC. CTP classification and BCLC stage accurately predicted the survival in these patients.

Terminology

Combination therapy with TACE and sorafenib is available for patients with unresectable, non-transplantable HCC. Adverse events secondary to both treatments are not unexpected and are comparable to what is reported in the literature.

Peer review

TACE alone or in combination with sorafenib is effective for the treatment of HCC. No statistical difference in survival was seen in the two treatment arms. No difference in outcome was seen after excluding patients with BCLC stage C and alpha fetoprotein > 400.

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BRIEF ARTICLE

In vivo assessment of intratumoral aspirin injection to treat hepatic tumors

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Abstract

AIM: To study the antineoplastic efficacy of 10% aspirin intralesional injection on VX2 hepatic tumors in a rabbit model.

METHODS: Thirty-two male rabbits (age: 6-9 wk; body weight: 1700-2500 g) were inoculated with VX2 hepatic tumor cells (10^4 cells/rabbit) *via* supraumbilical median laparotomy. On day 4 post-implantation, when the tumors were about 1 cm in diameter, the rabbits were randomly divided into the following groups (n = 8 each group) to assess early (24 h) and late (7 d) antineoplastic effects of intratumoral injection of 10% bicarbonate aspirin solution (experimental groups) in comparison to intratumoral injection of physiological saline solution (control groups): group 1, 24 h control; group 2, 24 h experimental; group 3, 7 d control; group 4, 7 d experimental. The serum biochemistry profile (measurements of glycemia, alkaline phosphatase, gamma-glutamyl transferase, aspartate aminotransferase, and alanine aminotransferase) and body weight measurements were obtained for all animals at the following time points: D0, before tumor implant; D4, day of treatment; D5, day of sacrifice for groups 1 and 2; D11, day of sacrifice for groups 3 and 4. Gross assessments of the abdominal and thoracic cavities were carried out upon sacrifice. The resected liver tissues, including hepatic tumors, were qualitatively (general morphology, signs of necrosis) and quantitatively (tumor area) assessed by histopathological analysis.

RESULTS: Gross examination showed no alterations, besides the left hepatic lobe tumors, had occurred in the thoracic and abdominal cavities of any animal at any time point evaluated. However, the features of the tumor foci were distinctive between the groups. Compared to the control groups, which showed normal unabated tumor progression, the aspirin-treated groups showed imprecise but limited tumor boundaries and a general red-white coloration (indicating hemorrhaging) at 24 h post-treatment, and development of yellow-white areas of a cicatricial aspect at 7 d after treatment. At all time points evaluated, all except one biochemical parameters tested within the reference range ($P > 0.05$); a significant increase was detected in the alkaline phosphatase level of the control group 3 on D11 ($P < 0.05$). At 24 h post-treatment, the aspirin-treated groups showed extensive coagulation necrosis accompanied by a remarkable absence of viable tumor foci; at 7 d after treatment, the tumors had completely disappeared in these animals and fibrous necrotic nodules had developed. In contrast, throughout the study course, the tumors of the control groups remained unchanged, showing tumor nodules without necrosis at the time point corresponding to 24 h post-treatment and increased amounts of tumor nodules at the time point corresponding to 7 d post-treatment. Quantitative analysis of the remaining tumor area revealed that the aspirin-treated groups had significantly smaller tumor foci at 24 h post-treatment (8.5% ± 0.7%) and

at 7 d after treatment (11.0% ± 4.2%), compared to those in the control groups (24 h: 98.5% ± 1.5% and 7 d: 94.0% ± 2.7%; both, $P < 0.005$).

CONCLUSION: Intralesional injection of a 10% aspirin solution causes destruction of VX2 hepatic tumors in rabbits without evidence of relapse at 7 d after treatment administration.

Key words: Intralesional injection; Intratumoral injection; Aspirin; Hepatic tumor; VX2; Rabbit; Antineoplastic; Therapy

Core tip: This experimental study employed the well-established VX2 hepatic tumor rabbit model to assess the antineoplastic efficacy of intratumoral aspirin injection. Analysis of early (24 h post-treatment) and late (7 d post-treatment) effects indicated that the therapy caused early tumor destruction, as evidenced by significant necrotic areas in histopathological analysis, without late recurrence, as demonstrated by hepatic tissue regeneration and restoration of liver function biochemical parameters.

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INTRODUCTION

Cancer is the leading cause of death in economically developed nations and the second leading cause of death in developing nations[1]. Myriad advances in detection and treatment modalities have led to decreases in the mortality rates for the most common cancers in the United States and other western countries (*i.e.,* lung, colorectal, female breast, and prostate); yet, many of these technologies have not yet reached the less developed and economically transitioning countries[2], where the rates of cancers are actually increasing. Thus, there remains a need for development of simple and effective therapeutic approaches; moreover, such novel therapies will be based upon the convenient and practical methodologies to determine a patient's prognosis that are currently in practice in the poorer and less technologically advanced clinics, such as histological detection of lymphatic compromise, local recurrence, tumor staging, and presence of distant metastases[3].

In general, cases of distant metastases frequently involve the liver, and these patients account for approximately 40% of the population diagnosed with terminal cancer[4]. Furthermore, colorectal cancer (a leading public health concern worldwide) is associated with high risk of liver metastasis; it has been estimated that nearly 50% of colorectal cancer patients develop liver metastasis at some point during the course of their disease[5].

Although many therapies targeting liver metastases are available[6], surgical resection remains the treatment option with the highest cure rate[6-9]. However, the curative efficacy is influenced by several features related to the metastasis itself (*i.e.*, number, location, and extent), the patient condition (*i.e.,* comorbidities, fitness for surgery/anesthesia), and the healthcare setting (*i.e.*, physician expertise, availability of technical and financial resources)[7,8]. As a consequence, curative surgery is not a feasible option for all patients; indeed, it has been estimated that up to 80% of patients with colorectal cancer liver metastases are not viable candidates for surgical removal[10].

Alternative non-surgical approaches are available for treating such patients[11,12]; the most common being physical ablative techniques (cryotherapy, radiotherapy, laser, and microwave) and chemotherapy[7-9], which have shown appreciable efficacy and safety profiles. However, clinical application of these approaches is still impacted by somewhat prohibitive cost and extent of involvement required of the patients (*e.g.,* several return visits for serial chemotherapy administrations), as well as adverse side effects (*e.g.,* emesis and anemia), some with life-threatening potential (*e.g.,* immune system suppression and anaphylactic shock). Thus, the need for a low-cost, simple antineoplastic treatment with good efficacy and low side effect profile has yet to be fulfilled[13].

Over the past few years, our research group has evaluated the cytolytic and antineoplastic potentials of acetylsalicylic acid (aspirin) and its derivatives[14-16]. The collective results from our *in vitro* (cultured tumor cell systems) and *in vivo* (animal-implanted tumors) analyses suggest that injecting aspirin directly into liver tumors may destroy the lesion with minimal or no adverse effects, either locally or systemically. Therefore, the current experimental study was designed to evaluate the therapeutic efficacy and safety of intratumoral aspirin injection using the well-established VX2 tumor rabbit model of hepatic metastases.

MATERIALS AND METHODS

Ethics statement

The study was conducted with pre-approval by the Ethics Committee of Botucatu Medical School at São Paulo State University (UNESP), Brazil. All procedures involving animals were carried out in accordance with the standards of published in the Care and Use of Laboratory Animals by the Institute for Laboratory Animal Research (1996) and the ethical principles of the Brazilian College on Animal Experimentation (COBEA).

Animal housing and tumor implantation

Thirty-two male New Zealand albino rabbits (6-9 wk-old, weighing 1700-2500 g) were housed under 12/12 h light-dark cycles with unrestricted access to standard rabbit

chow (Coelhil R® - Socil*:* Belo Horizonte, MG, Brazil) and water. Six hours prior to the tumor inoculation, the animals were fasted.

The rabbits were administered general anesthesia by intravenous injection of 3% sodium pentobarbital (30 mg/kg body weight). VX2 tumor cell suspension containing 10^4 cells (Boston University, MA, United States) were injected slowly into the left hepatic lobe using a 27-gauge needle *via* supra-umbilical median laparotomy, as previously described[17]. The laparotomy incision was closed by suturing with non-dissolving stitches (Ethicon mononylon 4-0; Johnson and Johnson, São José dos Campos, SP, Brazil).

Study design and intratumoral aspirin injection

Four days after the VX2 inoculation, when the tumors had reached about 1 cm in diameter[17], the rabbits were randomly divided into experimental and control groups (n = 16 each) for a second laparotomy to receive intratumoral injection of 10% aspirin or physiological saline solution, respectively. The 10% aspirin solution (pH: 7.27) was generated by diluting 5000 mg of acetylsalicylic acid (Pharma Nostra, Brazil) in 50 mL of 10% sodium bicarbonate solution. Treatments were administered as 0.5 mL aliquots of the experimental or control solution, as this volume was sufficient to infiltrate the entire hepatic lesion.

The experimental and control groups were further sub-divided into equal groups (n = 8 each) for analysis of early (24 h post-treatment) and late (7 d post-treatment) effects[14-16]. Thus, the four study groups were: group 1, 24 h non-treated VX2 tumor control; group 2, 24 h aspirin-injected VX2 tumor experimental; group 3, 7 d non-treated VX2 tumor control; group 4, 7 d aspirin-injected VX2 tumor experimental. At each group's end-of-treatment time, the animals were sacrificed by intravenous anesthesia overdose.

Monitoring of clinical evolution and effects on serum biochemistry markers of liver function

All animals underwent clinical evaluation to assess the disease evolution using objective parameters of post-surgical recuperation, such as resumption of feeding and activity. Effects on liver function were assessed by biochemical analysis of serum markers, including alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), aspartate aminotransferase (AST) and alanine aminotransferase (ALT). In addition, changes in body weight and glycemic status were recorded. Assessments were made at the following time points: D0, before tumor implant; D4, day of treatment; D5, day of sacrifice for groups 1 and 2; D11, day of sacrifice for groups 3 and 4.

Gross and microscopic analysis of liver and tumor specimens

Immediately upon anesthesia overdose, a third laparotomy was performed for specimen collection (all lesions were removed) and gross evaluation of the abdominal and thoracic cavities. The specimens were sectioned and prepared for histopathological analysis by bright field optical microscopy with hematoxylin-eosin staining, which was conducted by an experienced pathologist who was blinded to the study. Qualitative analysis was performed by analyzing the morphological features of tumor specimens. Quantitative analysis was performed by measuring the percentage of total liver tissue that was represented by tumor cells using the Optimas® 6.1 imaging software.

Statistical analysis

The significance of between-group differences in tumor tissue area (in mm^2) over time (in days) was assessed by the two-factor repeated measure ANOVA F test. Percentage data was analyzed using the non-parametric test for repeated measures. All statistical analyses were carried out by the SAS statistical software (version 9.2 for Windows; SAS Institute, United States). Statistical significance was indicated by 95%CI or P value of < 0.05.

RESULTS

Early effects of intratumoral aspirin injection

Clinical evolution, weight, glycemia, and liver function: At 24 h post-treatment, all animals in groups 1 and 2 presented good clinical evolution without any deaths. All biochemical parameters were within the normal range, and the differences between the control and experimental treatment groups did not reach statistical significance ($P > 0.05$).

Gross features of tumors and proximal tissues: Unlike the thoracic cavity, the abdominal cavity appeared to be remarkably affected by the experimental treatment. The animals in group 1 showed well-defined, solid, yellowy-white hepatic lesions, measuring between 0.9 and 1.2 cm in diameter, occurring as singlets in all rabbits. The animals in group 2 also developed singlet solid lesions, measuring between 0.8 and 0.9 cm, but with the distinctive gross features of imprecise but limited borders, red-white coloration (indicating hemorrhaging), more extensive involvement of the hepatic tissue, and a cystic aspect.

Histopathogical features of tumors: The livers from group 1 animals showed tumor nodules embedded throughout the normal hepatic tissue, and no necrotic areas (Figure 1A). The livers from group 2 animals also showed tumor nodules throughout the organ, but the hepatic parenchyma also showed extensive necrotic areas and hemorrhaging. In addition, intraparenchymal inflammatory infiltrates were observed, and there was a remarkable absence of viable tumor foci (Figure 1B).

The mean tumor area in livers from group 1 animals was significantly higher than that in the group 2 animals (98.5% *vs* 8.5%, $P = 0.0036$) (Table 1). This result clearly demonstrates the cytolytic effect of intratumoral aspirin injection.

Late effects of intratumoral aspirin injection

Clinical evolution, weight, glycemia, and liver function: Similar to the results at 24 h post-treatment, all ani-

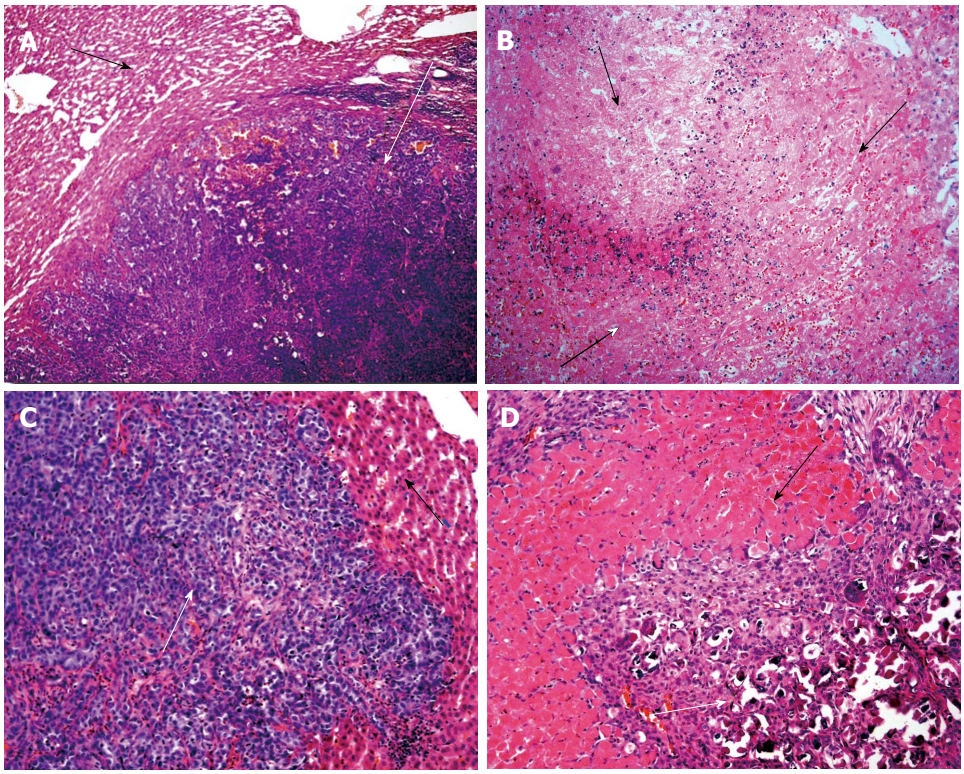

Figure 1 Photomicrograph of surgical specimen from liver VX2 tumor. A: 24 h after saline injection revealing a large tumor nodule (white arrow) surrounded by normal hepatic tissue with no signs of necrosis (black arrow). (Hematoxylin-eosin, original magnification × 100); B: 24 h after 10% aspirin solution injection showing extensive necrotic areas (black arrow). (Hematoxylin-eosin, original magnification × 200); C: 7 d after saline injection demonstrating a large viable tumor nodule (white arrow) with normal adjacent hepatic parenchyma (black arrow). (Hematoxylin-eosin, original magnification × 200); D: 7 d after 10% aspirin solution injection showing normal hepatic parenchyma (black arrow) surrounded by inflammatory infiltrate and fibrosis (white arrows). (Hematoxylin-eosin, original magnification × 100).

Table 1 Mean percentage values of remaining tumor area 24 h after treatment

Analysis	Groups		*P* value
	Group 1 (control)	Group 2 (aspirin)	
mean ± SD	98.5% ± 1.512%	8.5% ± 0.707%	0.0036
Median	98.5%	0%	
25 percentile	97.75%	0%	
75 percentile	100%	2%	

Table 2 Mean serum alkaline phosphatase levels at three times (D0, D4 and D11)

Variables	Times		
	Tumor implant (D0)	Day of treatment (D4)	7 d after treatment (D11)
Group 3	134[1]	129[2]	172[3]
Control - AP (U/L)			
Group 4 aspirin - AP (U/L)	128[1]	139[2]	117[3]
DMS value	15.3	14.6	19.5
CV value	9.39	8.69	11.7
F test value	0.63 (NS)	1.51 (NS)	31.9[4]

[1,2]No difference between them (DMS) at 5% significance level; [3]Difference between them (DMS) at 5% significance level; [4]Statistically significant. NS: Not significant; AP: Alkaline phosphatase; CV: Coefficient of variation; DMS: Tukey test.

mals in groups 3 and 4 presented good clinical evolution without any deaths at 7 d post-treatment. In additional, all animals experienced weight gain. All of the biochemical parameters measured were also within normal range, with the notable exception of ALP (Table 2). In group 3, the ALP level was enhanced over time (D4: 129 *vs* D11 172, $P < 0.05$). This effect was not observed in group 4, indicating that the antineoplastic effects of intratumoral aspirin injection also helped to restore liver function.

Gross features of tumors and proximal tissues: Similar to the results at 24 h post-treatment, the thoracic cavity appeared to be unaffected but the abdominal cavity appeared to be remarkably affected by the experimental treatment. The animals in group 3 showed solid, yellowy-white, nodular tumoral lesions, measuring between 1.2 and 1.6 cm in diameter; however, unlike the results at the early time point, each animal had developed multiple small punctiform lesions around the nodular tumoral lesion. These multiple lesions were restricted to the left hepatic lobe, and no other lesions were observed in the right lobe or in the rest of the abdominal cavity. The animals in group 4 showed small, superficial, yellowy-white lesions with cicatricial characteristics, measuring between 0.2 and 0.4 cm.

Table 3 Mean percentage values of remaining tumor area 7 d after treatment

Analysis	Groups		*P* value
	Group 3 (control)	Group 4 (aspirin)	
mean ± SD	94% ± 2.726%	11% ± 4.243%	0.0035
Median	94.5%	0%	
25 percentile	91.75%	0%	
75 percentile	96.25%	2%	

Histopathogical features of tumors: Similar to the results at 24 h post-treatment, the livers of group 3 animals showed well-defined tumor nodules throughout the hepatic tissues, and no necrotic areas (Figure 1C). In stark contrast to both the livers of group 3 and those from group 2 (at the 24 h post-treatment time point), the livers of group 4 showed no tumor nodules; only a few isolated tumor cells associated with the presence of fibrous necrotic nodules and actively proliferating normal hepatic ducts and cells were observed (Figure 1D).

The mean tumor area in livers from group 3 animals was significantly lower than that in the group 4 animals (94.0% *vs* 11.0%, $P = 0.0035$) (Table 3). This result clearly demonstrates the maintenance of the cytolytic effect of intratumoral aspirin injection.

DISCUSSION

The VX2 hepatic tumor rabbit model is a sufficiently accurate tool for experimental investigations of newly developed anti-tumor treatments, and has been successfully applied to research of adriamycin[18,19], microwave ablation[20], angiogenesis inhibitor[21], oxaliplatin[22,23], and interventional radiology[24,25]. To the best of our knowledge, however, the study described herein represents the first usage of this rabbit model to study the antineoplastic effects of intratumoral 10% aspirin injection.

The intratumoral aspirin injection produced good clinical and weight evolution in all animals, without any deaths, suggesting not only good therapeutic efficacy but also a good safety profile. In particular, no toxic or detrimental effects (either local or systemic) were observed. The absence of early effects on glycemia or liver function markers indicates that neither the implanted tumor cells nor the intratumoral aspirin treatment elicited any major functional alterations (that would be otherwise detectable by biochemical tests). However, a late effect on ALP levels was observed in untreated rabbits with hepatic tumors, suggesting that the tumorigenesis may induce intrahepatic cholestasis and bile duct compression[26]. The fact that this effect was absent in the aspirin-treated rabbits provides further evidence of this therapy's anti-tumor efficacy.

The lack of gross changes in the thoracic cavity (organs and serous membranes) of control animals suggests that the inoculated tumor cells did not undergo extensive or aggressive metastasis. In addition, the lack of gross changes (no signs of hemorrhaging or pulmonary condensation) in the thoracic cavity of experimental animals indicated that the intratumoral aspirin injection did not cause any damage to the proximal pulmonary tissues.

Obvious early differences in the gross features of livers with and without the aspirin treatment, including extensive coagulation necrosis in the treated hepatic parenchyma, minimal viable tumor foci, and quantifiable decrease in tumor cells, demonstrated rapid therapeutic efficacy. The low level of viable tumor cell foci present in the aspirin-treated livers may reflect usage of an insufficient injection volume or sub-optimal perfusion. Obvious late differences in the gross features of livers with and without the aspirin treatment indicated treatment-induced relief of tumoral lesions without evidence of recurrence. However, the aspirin-treated livers showed signs of fibrosis, suggesting that the remaining tumor tissue may differentiate to fibrotic scar tissue.

Recent studies with cultured human colorectal cancer cells have demonstrated the inhibitive activities of aspirin on proliferation and its inductive activities on apoptosis[27,28]. Still other *in vitro* studies have shown that aspirin can inhibit the growth of endometrial cancer cells[29], and induce apoptosis in human oral cancer cells[30] and in B cell chronic lymphocytic leukemia cells, *via* activation of caspases[31]. Moreover, aspirin pretreatment was found to augment TRAIL-induced apoptotic death in the human prostate adenocarcinoma line, LNCaP, and in the human colorectal carcinoma line, CX-1[32].

Regular intake of nonsteroidal anti-inflammatory drugs and cyclooxygenase (COX) inhibitors, such as aspirin, can reduce the risk of developing some cancers[33-35]. Considering that COX-2 overexpression is a frequent finding of many cancer specimens[36], we are intrigued by the idea that the direct application of aspirin to tumors may stimulate apoptosis and destroy the cancer cells through a mechanism involving inhibition of COX proteins.

Some limitations inherent to this study design may have impacted our results and must be considered with interpreting our findings. First, our study focused solely on one therapeutic agent, and no comparisons were made with similar substances, such as acetic acid. However, we previously demonstrated that aspirin has less toxicity than either aqueous phenol, acetic acid, or glycerine[14-16], and therefore we have focused our subsequent research on aspirin[37-39]. Second, we did not evaluate the pharmacological parameters of the aspirin treatment. Since acetylsalicylic acid is one of the best studied therapeutic substances[40-42], we chose to focus our current study on its antineoplastic benefit and safety as an intratumorally-delivered agent for liver cancer. Future experimental studies should not only be designed to overcome these limitations but also to include further long-term effects of this solution and delivery method prior to extending the analysis to humans in a clinical environment.

In conclusion, the rabbit VX2 hepatic tumor model was used to show that intratumoral injection of 10% aspirin can induce tumor destruction within 24 h after delivery, and that the antineoplastic effects were maintained out to 7 d post-treatment, with no signs of necrotic areas

or tumor nodules but with signs of hepatic tissue regeneration and fibrosis foci.

COMMENTS

Background

Colorectal cancer remains a major public health concern, especially in developing countries. Moreover, it is estimated that approximately one-half of patients with colorectal cancer will develop liver metastases during the disease course, yet only 20% of these individuals are good candidates for curative liver resection. As such, there is an urgent need for new treatment modalities that are simple, cost-effective, and efficacious.

Research frontiers

The current non-surgical treatment options for liver metastases have limited therapeutic efficacy, and are cost-prohibitive, inconvenient, and associated with detrimental side effects. Development of a new treatment modality that is technically simple, minimally-invasive, and able to be performed in a single (or minimal) application(s), such as intratumoral aspirin injection, will improve disease outcome among those patients who are not fit for curative resection.

Innovations and breakthroughs

Using the rabbit VX2 tumor model, intratumoral injection of aspirin was shown to safely, rapidly, and effectively induce tumor destruction followed by hepatic tissue regeneration and differentiation to fibrotic scar tissue.

Applications

These findings indicate the promise of a new therapeutic approach for managing unresectable liver metastases, which may be developed as a technically simple, low-cost, efficacious therapy for future clinical application.

Peer review

This is an interesting *in vivo* analysis demonstrating the antineoplastic effects of 10% aspirin in hepatic tumors, which was based on the VX2 rabbit hepatic tumor model. The content is of scientific interest has potential clinical relevance, but further research is needed prior to its application in humans in the clinical setting.

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BRIEF ARTICLE

Effect of dichloromethylene diphosphonate on liver regeneration following thioacetamide-induced necrosis in rats

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Abstract

AIM: To study the effect of dichloromethylene diphosphonate (DMDP), a selective Kupffer cell toxicant in reference to liver damage and postnecrotic liver regeneration in rats induced by sublethal dose thioacetamide (TA).

METHODS: Rats, intravenously (*iv*) pre-treated with a single dose of DMDP (10 mg/kg), were intraperitoneally (*ip*) injected with TA 6.6 mmol/kg (per 500 mg/kg body weight). Hepatocytes were isolated from rats at 0, 24, 48 and 72 h following TA intoxication and blood and liver samples were obtained. To evaluate the mechanisms involved in the postnecrotic regenerative state, DNA distribution and ploidy time course were assayed in isolated hepatocytes. Circulating cytokine tumor necrosis factor-alpha (TNF-α) was assayed in serum and determined by reverse transcriptase-polymerase chain reaction in liver extract.

RESULTS: The effect of DMDP induced noticeable changes in postnecrotic regeneration, causing an increased percentage of hepatocytes in the cell cycle S phase. The increase at 24 h in S_1 population in rats pretreated with DMDP + TA was significantly ($P < 0.05$) different compared with that of the TA group (18.07% *vs* 8.57%). Hepatocytes increased their proliferation as a result of these changes. Also, TNF-α expression and serum level were increased in rats pre-treated with DMDP. Thus, DMDP pre-treatment reduced TA-induced liver injury and accelerated postnecrotic liver regeneration.

CONCLUSION: These results demonstrate that Kupffer cells are involved in TA-induced liver, as well as in postnecrotic proliferative liver states.

Key words: Dichloromethylene diphosphonate; Kupffer cells; Thioacetamide; Hepatotoxicity; Cell cycle

Core tip: Over the last 20 years, liposomes, useful models for cell membranes, have become a powerful research tool whose study has resulted in many advances in cell physiology. When encapsulated in liposomes, dichloromethylene diphosphonate, a selective Kupffer cell toxicant, completely eliminates large Kupffer cells from the liver, allowing us to elucidate the role of these macrophages in total damage induced by hepatotoxic compounds such as thioacetamide.

Bautista M, del Rio MÁG, Benedí J, Sánchez-Reus MI, Morales-González JA, Téllez-López AM, López-Orozco M. Effect of dichloromethylene diphosphonate on liver regeneration following thioacetamide-induced necrosis in rats. *World J Hepatol* 2013; 5(7): 379-386 Available from: URL: http://www.wjgnet.com/1948-5182/full/v5/i7/379.htm DOI: http://dx.doi.org/10.4254/wjh.v5.i7.379

INTRODUCTION

Dichloromethylene diphosphonate (DMDP) is clinically employed for the treatment of osteolytic bone diseases. When encapsulated in liposomes, DMDP is a selective Kupffer cell toxicant that completely eliminates large Kupffer cells from the liver, resulting in their damage and apoptosis[1]. Degree of depletion depends on the injection route and amount of injected DMDP liposomes. In the majority of studies, not only Kupffer cells, but also splenic macrophages have been depleted by a single intravenous (*iv*) injection of DMDP. Kupffer cells, due to the macrophages residing in the liver sinusoids, are the first macrophage population to come into contact with drugs. These cells are anchored to the endothelium in the lumen of the sinusoids[2]. Kupffer cells exhibit intra-acinar heterogeneity because those located in the periportal area are larger and exhibit higher phagocytic activity compared with those localized in the perivenous area[3]. It is well known that the function of these cells (cytokine and protease release, superoxide anion production, *etc*.) plays an important role in the pathogenesis induced by hepatotoxic compounds[4,5]. DMDP is most likely protective because it prevents the release of inflammatory cytokines and toxic oxygen radicals produced by activated Kupffer cells[6,7].

Thioacetamide (TA) is a potent hepatotoxic agent that, when administered at 500 mg/kg body weight doses to rats, gives rise to severe hepatocellular perivenous necrosis[8,9]. The selective destruction of perivenous hepatocytes and the proliferative state of liver cells that immediately follows were employed in the present study as an experimental model by means of which to study the hepatic response against the aggressive attack of a hepatotoxic drug. Thus, this response may be considered from two perspectives: that of hepatocellular necrosis and that of the postnecrotic hepatocellular regeneration linked with restoration of liver function[10,11].

Kupffer cells are also the major source of mitogens such as tumor necrosis factor-alpha (TNF-α) in liver[12,13]. TNF-α is a multifunctional cytokine that in the liver acts as a mediator of the acute phase response and is a cytotoxic agent in many types of hepatic injury. Some authors have suggested that TNF-α may be necessary for hepatocyte proliferation[14]. The observation that TNF-α is required for liver regeneration is surprising because TNF-α is a proinflammatory cytokine and an acute phase response mediator[15]. The proliferative and anti-apoptotic effect of this cytokine appears to take place only under special conditions, such as those existing after partial hepatectomy. Although TNF-α appears to be beneficial and required for liver regeneration after partial hepatectomy, the need for this factor has not been as clearly established after liver injury, a more common regenerative stimulus. In fact, a number of studies have suggested that TNF increases liver injury after toxic damage[16,17]. Moreover, Fujita *et al*[18] demonstrated that the absence of TNF-α does not impair liver regeneration.

The purpose of the present study was to elucidate the role of Kupffer cells in regeneration after liver injury, specifically blocking Kupffer cell function by DMDP. The proliferative postnecrotic response was assayed by evaluating ploidy and DNA distribution in the cell cycle phases in isolated hepatocytes by flow cytometry.

MATERIALS AND METHODS

Reagents

DMDP (dichloromethylene diphosphonate) was provided by Roche Diagnostics (Mannheim, Germany), phosphatidylcholine by Lipoid EPC, LIPOID (Ludwigshafen, Germany) and monoclonal ED-1 (MCA1018G) and monoclonal ED-2 antibodies were provided by Serotec, Hilversum (The Netherlands). Enzymes were obtained from Boehringer Mannheim (Germany). Substrates and coenzymes were from Sigma Chemical Co. (St. Louis, MO, United States). Standard analytical grade laboratory reagents were obtained from Merck (Darmstadt, Germany).

Liposome-encapsulated DMDP

Liposomal clodronate was prepared as previously described[19]. Briefly, 86 mg phosphatidylcholine and 8 mg cholesterol were dissolved in chloroform in a round-bottom flask. The thin film that formed on the interior of the flask after high vacuum rotary evaporation was dispersed by gentle rotation under low vacuum conditions for 10 min in 10 mL phosphate buffered saline (PBS) (control liposomes) or in 10 mL of a 0.6 mol/L DMDP (2.5 g DMDP in 10 mL distilled water and clodronate-containing liposomes). After swelling, sonication and washing in PBS, the liposomes were resuspended in 4 mL PBS. The resulting liposomal formulation contained clodronate at a concentration of 0.7 mol/L.

Animal treatment and sample processing

Two month old male Wistar rats (weighing 200-220 g) were obtained from the Bioterio, Instituto de Ciencias de la Salud, Universidad Autónoma del Estado de Hidalgo (UAEH), Mexico, and acclimated to our animal room for 2 wk, during which time the rats were supplied with food (Purina de México, S.A.) and water ad libitum, exposed to a 12 h light-dark cycle, and administered intraperitoneally (*ip*) with a single necrogenic dose of thioacetamide (TA) 6.6 mmol (500 mg/kg body weight) (TA) freshly dissolved in 0.9% NaCl. The TA dose was chosen as the highest dose with survival of > 90%[20,21]. Experiments were performed on two different groups.

Rats were treated with a single dose of TA and rats pre-treated with DMDP 24 h prior to TA (DMDP + TA). DMDP encapsulated in liposomes was injected into tail vein (10 mg/kg). Untreated animals received 0.5 mL of 0.9% NaCl. Rats were cervically dislocated and blood and liver samples were obtained and processed as previously described[21]. Blood was collected from hearts and maintained at 4 ℃ for 24 h, centrifuged at 3000 *g* for 15 min, and serum was obtained as the supernatant. Hepatocytes were isolated from rats by the classic perfusion method[22] at 0, 24, 48 and 72 h following TA (24 h). The viability of isolated hepatocytes (> 90%) was assessed by trypan blue exclusion as previously described[10].

Each experiment was performed in duplicate on four different animals and following the International Criteria of Experimental Animals outlined in Care and Use of Laboratory Animals, DHEW Publication No. (NIH) 85-23, 1985, and all procedures involving experimental animals were conducted according to our Federal Regulations for Animal Experimentation and Care (Ministry of Agriculture; SAGAR, Mexico) and The Guiding Principles in the Use of Animals in Toxicology adopted by the Society of Toxicology in 1989.

Determination of parameters of injury and TNF-α in serum

Enzymatic determinations were carried out in serum under optimal conditions of pH, temperature, substrate and co-factor concentrations. Aspartate aminotransferase (AST) and isocitrate dehydrogenase (ICDH) were determined in serum as a biochemical indicator of hepatocellular necrosis according to the manufacturer's protocol. AST (EC 2.6.2.1) activity was assayed following the method of Rej and Horder[25]. ICDH (E.C 1.1.1.39) was determined as described previously[26]. Concentrations of immunoreactive TNF-α was determined by the enzyme-linked immunosorbent assay (ELISA) system (Amersham Pharmacia Biotech) according to the manufacturer's protocol. In brief, the extracted plasma was reacted with the assay reagents in the TNF-α kit and analyzed spectrophotometrically at 450 nm absorbance. TNF-α levels were calculated from kit standards and expressed as pg/mL of plasma.

RNA extraction and reverse transcriptase-polymerase chain reaction analysis of TNF-α

Total RNA was isolated from rat liver following the guanidinium thiocyanate/phenol reagent method[27]. For reverse transcriptase-polymerase chain reaction (RT-PCR), total RNA (1 μg) was subjected to random primer first-strand complementary DNA (cDNA) synthesis in 40 μL reactions composed of 50 mmol/L Tris-HCl, 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 10 mmol/L DTT, 1 mmol/L dNTP (each), 50 ng of random hexamer and 0.5 IU/μL Mo-Mu-LV reverse transcriptase (Super-Script Pre-Amplification System; Gibco-BRL, Life Technologies). The reactions were incubated for 60 min at 42 ℃ and terminated at 65 ℃ for 15 min. First-strand cDNA were subsequently amplified by PCR; β-actin cDNA was utilized as an internal control. Sequences of the primers were as follows: TNF-α sense: 5'-TGG CCC AGA CCC TCA CAC TC-3'; TN-α antisense: 5'-CTC CTG GTA TGA AAT GGC AAA TC-3'; β-actin sense: 5'-TAC AAC CTC CTT GCA GCT CC-3'; and β-actin antisense: 5'-GGA TCT TCA TGA GGT AGT CAG TC-3'. The PCR reaction mixture contained PCR buffer [20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl], 1.5 mmol/L $MgCl_2$, 100 mmol/L dNTP (each), 0.4 mmol/L primers and 0.0025 U/μL of Taq polymerase in a final volume of 50 μL. Number of PCR cycles was adjusted to avoid saturation of the amplification system [at 94 ℃ for 1 min, 59 ℃ for 1 min and 72ºC for 1 min (35 cycles) for TNF-α, and at 94 ℃ for 30 s, 58 ℃ for 45 s and 72 ℃ for 30 s (24 cycles) for β-actin], with a final elongation at 72 ℃ for 10 min. Amplification products were visualized on 1.8% agarose gels containing ethidium bromide (1 μg/mL), TNF-α product, 281 bp, and β-actin product, 630 bp. A 100 bp DNA ladder was used as a marker. The products were quantified by laser densitometry.

Flow cytometry analysis of DNA content

DNA content was obtained from 10^6 isolated viable hepatocytes stained with propidium iodide following the multistep procedure of Vindeløv *et al*[28]. The fluorescence emitted from the DNA-propidium iodide complex was assayed in a FACScan flow cytometer (Becton-Dickinson) in the FL2-A channel. A double discriminator module was employed to distinguish between signals deriving from a single nucleus and nuclear aggregation products. Data analysis was carried out by evaluation of single inputs (10^4 nuclei/assay) and was expressed as the percentage of DNA distribution in cell cycle phases G_0/G_1 (2N), S_1, G_2 + M (4N), S_2, $(G_2 + M)_2$ (8N) and hypodiploid peak (< 2N).

Statistical analysis

The results were calculated as the mean ± SD of four experimental observations in duplicate (four animals). Differences between groups were analyzed by analysis of variance (ANOVA) following Snedecor F ($\alpha = 0.05$). The Student's *t* test (statistical significance $P < 0.05$) was performed for statistical evaluation as follows: (1) all values against their control; and (2) differences between two groups: DMDP + TA *vs* TA.

RESULTS

Effect of DMDP on parameters of liver necrosis

Liver damage induced by xenobiotics is characterized by the release in serum of hepatic enzymes due to the necrosis of hepatocytes. AST is randomly distributed in the hepatic acinus and is the enzyme activity utilized as the marker of necrosis. The increase in AST and ICDH in serum reached the maximum at 24 h (Figure 1). The extent of TA-induced necrosis was detected by a peak of 30 and 15 times baseline values for AST and ICDH activity, respectively. When rats were pre-treated with DMDP, the

Table 1 Quantitative analysis of the DNA ploidy in hepatocytes of adult rats following different treatments

Group	Hypodiploid (< 2N)	Diploid (2N)	S1 Phase (2N → 4N)	Tetraploid (4N)	S2 Phase (4N → 8N)	Octoploid (8N)
Control	0.98	12.3	2	75.31	2.48	3.97
Control DMDP	0.74	18.86	0.98	71.09	5.6	2.67
TA 24 h	1.61	41.74[a]	8.57[a]	39.0[a]	7.82[a]	0.9
TA-DMDP 24 h	2.01	25.45[a,c]	18.07[a,c]	49.20[a,c]	3.92	0.76
TA 48 h	1.59	52.87[a]	11.95[a]	22.86[a]	10.18[a]	0.2
TA-DMDP 48 h	2.35	42.77[a]	14.92[a]	28.0[a]	10.25[a]	1.7
TA 72 h	3.78[a]	47.61[a]	7.21[a]	35.6[a]	4.29[a]	1.12
TA-DMDP 72 h	2.65[a,c]	45.99[a]	1.44[c]	41.71[a]	5.98	1.83

The values are expressed as the percentage of DNA in the following: hypodiploid population (< 2N); diploid population, 2N (cells in G0-G1); tetraploid population, 4N (cells in G2 + M); octaploid population, 8N, and cells synthesizing DNA, S1 phase (from G1-G2, 2N→4N) and S2 phase (from 4N→8N). Data are expressed as the means of four experimental observations (four rats) ± SD, [a]$P < 0.05$ *vs* the untreated control group; [c]$P < 0.05$ *vs* changes due to dichloromethylene diphosphonate (DMDP). TA: Thioacetamide.

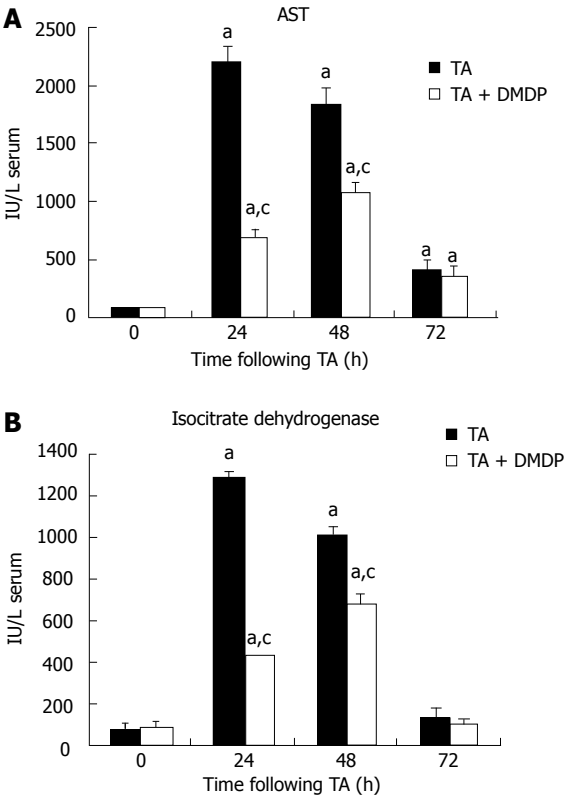

Figure 1 Enzymatic activity after dichloromethylene diphosphonate pre-treatment in rats intoxicated with one sublethal dose of thioacetamide. A: Effect of dichloromethylene diphosphonate (DMDP) pre-treatment on aspartate aminotransferase (AST) activity in the serum of rats intoxicated with one sublethal dose of thioacetamide (TA); B: Illustrates the effect of DMDP pre-treatment on isocitrate dehydrogenase activity in the serum of rats intoxicated with one sublethal dose of TA. Samples were obtained at 0, 12, 24, 48 and 72 h following TA 6.6 mmol (per 500 mg/kg body weight). The results, expressed as nmol per min per mL of serum, are the mean ± SD of four determinations in duplicate from four rats. [a]$P < 0.05$ *vs* the respective control, [c]$P < 0.05$ *vs* differences due to DMDP.

24 h peaks were reduced to 30% and 40%, respectively. However, at 48 h of intoxication, the DMDP-associated difference was 58% for AST activity, which indicates that DMDP delays TA-induced liver injury because maximal necrosis appeared at 48 h of intoxication. No effects were detected on serum activities when empty liposomes were administered (data not shown).

Effect of DMDP pre-treatment on the time course of genomic DNA ploidy and distribution in hepatocytes isolated from TA-treated rats

Table 1 shows the percentages of cell cycle populations related with ploidy and DNA content, as associated with histograms determined on the basis of fluorescence emission at 623 nm by the DNA propidium iodide complex. Following TA, liver cells exhibit marked variations in the pattern of DNA distribution, which can be summarized as a sharp decrease at 48 h in tetraploid population parallel to an increase in diploid population, followed by restoration to nearly normal values at 72 h. It can also be observed how the S_1 population is increased from 24 h, reaching maximal increase at 48 h. When rats were pre-treated with DMDP, variations in the pattern of DNA distribution is very similar to that observed in the TA group. However, we are able to detect an important difference: the highest increase in S_1 population is reached at 24 h (18.07% *vs* 8.57%) instead of at 48 h; thus, the proliferative state in hepatocytes is reached 24 h prior to that obtained in single dose TA-treated rats. No changes were detected in DNA ploidy when empty liposomes were administered (data not shown).

Effect of DMDP pre-treatment on serum TNF-α levels and expression in liver of rats following liver intoxication with TA

TNF-α is a multifunctional cytokine that, in the liver, acts as a mediator of the acute phase response and is a cytotoxic agent in many types of hepatic injury. TNF-α determination was performed in serum and liver. In TA-intoxicated rat serum, the level of this cytokine increased at 24 h of intoxication and when DMDP was pre-administered; this increase was significant (Figure 2).

Figure 2B and C depict the levels of TNF-α messenger RNA (mRNA) assayed by RT-PCR. As observed in

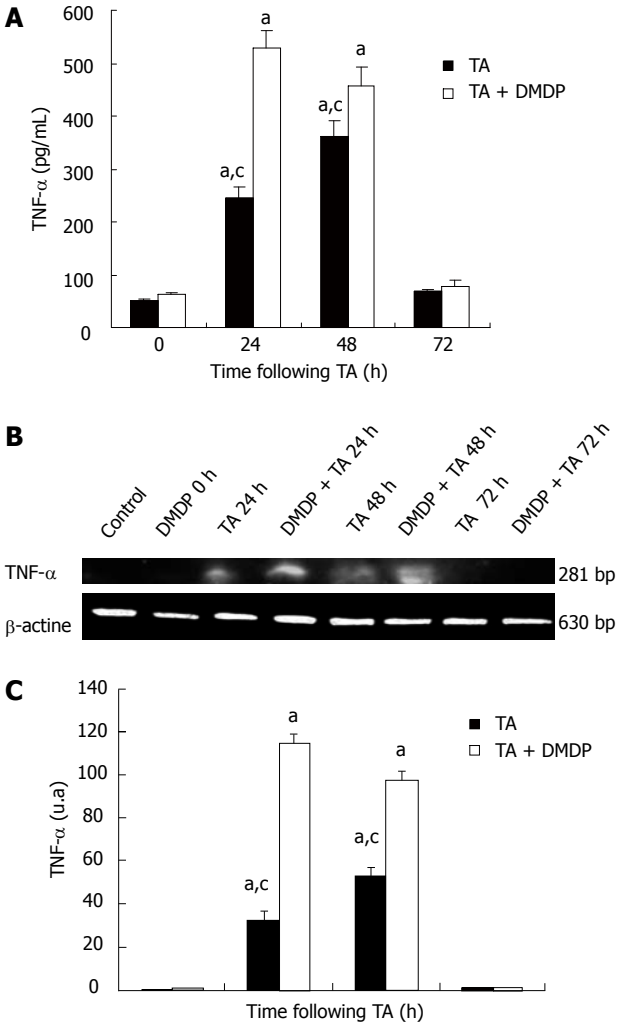

Figure 2 Effects of dichloromethylene diphosphonate in protein levels, gene expression and messenger levels tumor necrosis factor-alpha. A: Effects of dichloromethylene diphosphonate (DMDP) pre-treatment on serum tumor necrosis factor-alpha (TNF-α) levels determined by enzyme-linked immunosorbent assay tests on serum samples. Columns and vertical bars represent mean ± SD evaluated in four determinations from four rats. $^{a}P < 0.05$ *vs* control group; $^{c}P < 0.05$ *vs* the DMDP-treated group; B: Effects of DMDP in gene expression profile of TNF-α assayed by reverse transcriptase-polymerase chain reaction analysis; C: Illustrates the effect of DMDP pre-treatment on the levels of TNF-α messenger RNA (mRNA) in liver homogenates of rats intoxicated with a sublethal dose of thioacetamide (TA). Samples were obtained at 0, 24, 48 and 72 h. The results, expressed in arbitrary units, are the mean ± SD of four determinations from four rats. $^{a}P < 0.05$ *vs* the respective control, $^{c}P < 0.05$ *vs* differences due to DMDP.

serum levels of TNF-α , mRNA follow the same pattern, which corroborates with the results obtained by ELISA.

DISCUSSION

Macrophages such as Kupffer cells in liver are multifunctional cells. They are involved in host defense mechanisms and possess a regulatory role in many biomedical processes. Their selective depletion[6], employing liposome-encapsulated drugs, forms a widely accepted approach to studying their functional aspects *in vivo*. There is evidence that liposome-mediated DMDP delivery actually depleted Kupffer cell in rat liver. We and others[7,29] found this "suicide" approach highly effective in depletion of Kupffer cell in liver tissue.

On the other hand, TA-induced liver injury is a well-established area of considerable pharmacological interest because reactive oxygen species (ROS) and free radicals, generated in microsomal drug oxidation, participate in the mechanisms of cell death[20,21,30]. Xenobiotics may act directly on hepatocytes, causing toxicity by interacting with target molecules, and may also act indirectly by means of activating phagocytic cells. The active phagocytes participate in the pathogenesis of tissue injury by releasing, among others, inflammatory cytokines that upregulate the expression of adhesion molecules. Tissue damage initiates an inflammatory response characterized by an accumulation of neutrophils at the site of injury[31].

Kupffer cells and infiltrating neutrophils contribute to liver injury in different experimental models of hepatotoxicity[32-34]. In our experiments, DMDP significantly attenuates TA-induced liver damage. Blockade of Kupffer cell function by DMDP appears to result in a disruption of a part of the sequence of events leading to hepatotoxicity.

In addition, the role of DMDP in TNF-α expression by Kupffer cells has been widely debated. Depletion of Kupffer cells, the major source of TNF-α production in liver, should give rise to a decrease in serum and in the mRNA TNF-α level in liver, a fact that has been described and corroborated by several authors[5,35,36]. However, other authors have reported opposite data[37,38] after partial hepatectomy in rats pre-treated with gadolinium, another inhibitor of Kupffer cells. Additionally, depletion of Kupffer cells with DMDP appears to increase hepatocyte proliferation and liver regeneration following partial hepatectomy; however, the responsible mechanism remains unknown.

On the other hand, it has already been reported that DMDP protects the liver from a number of toxicants that require biotransformation to elicit toxicity[39,40]. Badger *et al*[41] demonstrated in hepatocytes isolated from DMDP pre-treated rats that CYP-450 activity was reduced and the susceptibility of hepatocytes was altered. It has also been shown that hepatic injury induced by ischemia/reperfusion is modulated by the Kupffer cells[42].

In previous reports, we described that when TA was administered to rats, necrosis developed and peaked at 24 h of intoxication and that a synchronous proliferative response was immediately initiated, reaching a peak of DNA synthesis at 48 h. Postnecrotic proliferative response after experimental liver cell death constitutes an interesting area in which to study the factors involved in the regulation of hepatocyte proliferation.

Regarding postnecrotic regeneration, the peak of DNA synthesis was similar in both groups, although it is noteworthy that initial DNA synthesis levels were significantly higher due to the effect of DMDP, indicating that in our experiments, this compound also exerts mitogenic action, which can lead to liver hyperplasia.

After depleting Kupffer cells with DMDP, we explain that elevation of serum TNF-α levels and enhanced mRNA levels in the liver by hepatic cells other than Kupffer cells may contribute to cytokine synthesis or that TA-inducible cells residing in the liver contribute to cytokine levels in plasma. Endothelial cells may be a hepatic source of cytokines because this cell type readily responds to TA stimulation[35,43] and may not be affected directly by clodronate.

Following TA, liver cells exhibit marked variations in the DNA distribution pattern, which can be summarized as a sharp decrease at 48 h in tetraploid population parallel to an increase in diploid population, followed by restoration to nearly normal values at 72 h. It can also be observed how the S_1 population increases from 24 h, reaching the maximum at 48 h. When rats were pretreated with DMDP, variations in the pattern of DNA distribution are very similar to those observed in the TA group. However, we can detect an important difference: the highest increase in S_1 population is reached at 24 h (17.17% *vs* 10.01%) instead of at 48 h; thus, the proliferative state in hepatocytes is reached 24 h prior to that obtained in rats treated with the single dose of TA.

Our results clearly indicate that administration of DMDP + TA in rats results in stimulated tissue repair. From these results, we are able to speculate that Kupffer cells may play a crucial role in inducing DNA synthesis by secreting the priming factors (TNF-α) in the early phase of oval cell-mediated liver regeneration[44].

We conclude that DMDP pre-treatment significantly attenuates TA-induced hepatotoxicity. These results demonstrate that Kupffer cells are involved in TA-induced liver, as well as in postnecrotic proliferative liver states.

Modulation of Kupffer cell function by DMDP may serve as a potential target for therapeutics and could be useful for preventing drug-induced liver damage.

COMMENTS

Background

Thioacetamide (TA)-induced liver injury is a well-established area of considerable pharmacological interest because reactive oxygen species (ROS) and free radicals generated in microsomal drug oxidation participate in the mechanisms of cell death. In the present study, TA-induced hepatotoxicity was used to investigate the effect of a single dose of dichloromethylene diphosphonate (DMDP) (clinically employed for the treatment of osteolytic bone diseases); but in the present study, when encapsulated in liposomes, DMDP is a selective Kupffer cell toxicant.

Research frontiers

The aim of this study was to elucidate the role of Kupffer cells in regeneration after liver injury, specifically blocking Kupffer cell function by DMDP. The effect was assayed on an experimental model of liver injury induced by a single sublethal dose of TA.

Innovations and breakthroughs

Macrophages such as Kupffer cells in the liver are multifunctional cells. They are involved in host defense mechanisms and possess a regulatory role in many biomedical processes. Their selective depletion, utilizing liposome-encapsulated drugs, forms a widely accepted approach of studying their functional aspects *in vivo*.

Applications

As it is generally accepted that Kupffer cell function is involved in the severity of drug-induced liver damage and that DMDP induces a selective blockade of Kupffer cell function when administered intravenously, the purpose of the present study was to elucidate the role of Kupffer cells in regeneration after liver injury, opening a window to novel therapeutic strategies.

Terminology

Liposomes can be used for intracellular drug delivery into macrophages. In the present study, the authors utilized a liposome-mediated macrophage "suicide" technique based on intraphagocytic accumulation of the liposomes delivered.

Peer review

This is a nice experimental study showing that Kupffer cells play an important role in experimental hepatotoxicity by TA and in the regenerating process. The paper is well written.

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BRIEF ARTICLE

Hepatitis B virus reactivation in hepatitis B virus surface antigen negative patients receiving immunosuppression: A hidden threat

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Abstract

AIM: To present the characteristics and the course of a series of anti- hepatitis B virus core antibody (HBc) antibody positive patients, who experienced hepatitis B virus (HBV) reactivation after immunosuppression.

METHODS: We retrospectively evaluated in our tertiary centers the medical records of hepatitis B virus surface antigen (HBsAg) negative patients who suffered from HBV reactivation after chemotherapy or immunosuppression during a 3-year period (2009-2011). Accordingly, the clinical, laboratory and virological characteristics of 10 anti-HBc (+) anti-HBs (-)/HBsAg (-) and 4 anti-HBc (+)/antiHBs (+)/HBsAg (-) patients, who developed HBV reactivation after the initiation of chemotherapy or immunosuppressive treatment were analyzed. Quantitative determination of HBV DNA during reactivation was performed in all cases by a quantitative real time polymerase chain reaction kit (COBAS Taqman HBV Test; cut-off of detection: 6 IU/mL).

RESULTS: Twelve out of 14 patients were males; median age 74.5 years. In 71.4% of them the primary diagnosis was hematologic malignancy; 78.6% had received rituximab (R) as part of the immunosuppressive regimen. The median time from last chemotherapy schedule till HBV reactivation for 10 out of 11 patients who received R was 3 (range 2-17) mo. Three patients (21.4%) deteriorated, manifesting ascites and hepatic encephalopathy and 2 (14.3%) of them died due to liver failure.

CONCLUSION: HBsAg-negative anti-HBc antibody positive patients can develop HBV reactivation even 2 years after stopping immunosuppression, whereas prompt antiviral treatment on diagnosis of reactivation can be lifesaving.

Key words: Immunosuppression; Hepatitis B; Anti-hepatitis B virus core antibody positivity; Occult hepatitis B virus infection; Rituximab

Core tip: Patients with occult or resolved hepatitis B

undergoing chemotherapy or immunosuppression are potentially at risk of hepatitis B virus (HBV) reactivation which can be disastrous since it can lead to acute liver failure and death. In this report, we describe the characteristics and outcome in one of the larger series of patients (n = 14) with occult or resolved HBV who experienced HBV reactivation after receiving immunosuppression though they were initially HBV surface antigen-negative. Most of patients had received rituximab. We showed that these patients can develop severe HBV reactivation even 2 years after stopping immunosuppression, whereas prompt antiviral treatment on diagnosis of reactivation can be lifesaving.

Zachou K, Sarantopoulos A, Gatselis NK, Vassiliadis T, Gabeta S, Stefos A, Saitis A, Boura P, Dalekos GN. Hepatitis B virus reactivation in hepatitis B virus surface antigen negative patients receiving immunosuppression: A hidden threat. *World J Hepatol* 2013; 5(7): 387-392 Available from: URL: http://www.wjgnet.com/1948-5182/full/v5/i7/387.htm DOI: http://dx.doi.org/10.4254/wjh.v5.i7.387

INTRODUCTION

Hepatitis B virus (HBV) is a common cause of liver disease, affecting more than 240 million people worldwide[1]. HBV carriers are traditionally identified by the detection of HBV surface antigen (HBsAg) in their blood. During the past 15 years with the availability of highly sensitive molecular methods, persistence of HBV genomes in HBsAg negative individuals has been clearly proven termed occult HBV infection (OBI).

Accordingly, OBI is defined by the presence of HBVDNA in the liver tissue or also in the serum of HBsAg negative individuals who are either anti-HBc antibodies (Abs) and/or anti-HBs Abs positive or even have negative serological markers[2-4]. OBI is mainly related to a strong suppression of the viral activity in which the host's immune surveillance is likely to play a major role. Therefore, patients with OBI undergoing strong immunosuppression are potentially at risk of HBV reactivation, a common phenomenon in HBsAg-positive hematogical or oncological patients[5,6]. HBV reactivation has also been reported in patients with OBI treated with synthetic disease modifying antirheumatic drugs (DMARDs) and/or high-dose prednisolone for rheumatic diseases[7-13]. Especially in the era of targeted immune modulators (commonly referred to as biological response modifiers or "biologics"), which cause profound immunosuppression and are used in the treatment of immunological, inflammatory as well as hematological/oncological diseases, the risk becomes even greater[14].

The European Association for the Study of the Liver clinical practice guidelines for the management of chronic HBV infection in HBsAg-negative patients with positive anti-HBc Abs who receive chemotherapy and/or immunosuppression suggest HBVDNA determination in the serum and if undetectable, strict follow-up consisting of alanine aminotransferase (ALT) and HBVDNA testing[15]. Treatment with potent antivirals with high barrier to resistance (*i.e.*, entecavir or tenofovir) is recommended upon confirmation of HBV reactivation before ALT elevation[15]. However, there are no surrogates or prognostic markers for impending HBV reactivation, making the follow-up of these patients difficult, since cost-effectiveness of serial and frequent HBVDNA testing has not been documented. For these reasons it is urgent to define the characteristics of these patients with OBI who experience HBV reactivation, when they receive immunosuppression as well as the features of the HBV reactivation itself.

Accordingly, in this case-study we describe the course of 14 patients with OBI who received intense immunosuppression with various biological or non-biological immune modifying agents for diverse pathological entities and experienced HBV reactivation.

MATERIALS AND METHODS

Patients

We retrospectively evaluated in our tertiary centers (Aristotle University Medical School and University of Thessaly Medical School) the medical records of HBsAg seronegative patients who suffered HBV reactivation after chemotherapy or immunosuppression during a 3-year period (2009-2011). Accordingly, we identified 14 patients with occult [anti-HBc (+)/anti-HBs (-)/HBsAg (-), n = 10] or resolved [anti-HBs (+)/antiHBc (+)/HBsAg (-), n = 4] HBV infection, who developed HBV reactivation after the initiation of chemotherapy or immunosuppressive treatment. The clinical, laboratory and virological characteristics of the patients were recorded. The mean follow-up of the patients from the time of the diagnosis of HBV reactivation was 8 (range 1-60) mo. Although there are no clear diagnostic criteria for HBV reactivation, we defined HBV reactivation as seroconversion from HBsAg negative to HBsAg positive with serum HBV DNA turning from negative to positive[16]. Quantitative determination of HBV DNA during reactivation was performed in all cases by a quantitative real time polymerase chain reaction kit (COBAS Taqman HBV Test; cut-off of detection: 6 IU/mL). All patients were anti- hepatitis C virus negative, anti-hepatitis A virus IgG positive, anti-HIV negative at baseline (before the initiation of immunosuppression) as well as at the time of HBV reactivation.

RESULTS

The 10 anti-HBc (+)/anti-HBs (-)/HBsAg (-) patients were all males, whereas 2 out of 4 anti-HBc (+)/anti-HBs (+)/HBsAg (-) patients were females. The median age at diagnosis of HBV reactivation was 74.5 (range 53-82) years, while the median age of the diagnosis of their primary disease and initiation of immunosuppression was 73 (range 49-80) years. The primary diagnosis

Table 1 Characteristics and outcome of the ten hepatitis B surface antigen (-)/anti-hepatitis B virus core antibody (+)/anti-hepatitis B virus surface antigen (-) patients (at baseline) who experienced hepatitis B virus reactivation after immunosuppressive therapy

No.	Age, yr (at reactivation)	Sex	Disease	Type of IMS	Months from start of IMS to HBV reactivation	Months from last treatment cycle to diagnosis of HBV reactivation	HBV-DNA (IU/mL) at diagnosis of HBV reactivation	ALT/Bil/INR at diagnosis of HBV reactivation	Antiviral treatment	Serology at end of follow-up	Outcome
1	77	M	NHL	C, Flud, R	6	3	> 17857000	1472/3.22/1.3	Tenofovir	Anti-HBc (+)	Alive, response[1]
2	71	M	NHL	CHOP-R	8	1	487934	871/8.8/0.87	Entecavir	Anti-HBc (+)	Liver related death
3	74	M	NHL	C, Flud, R	6	1	1310000	73/0.7/0.97	Tenofovir	NA	Alive, response[1]
4	76	M	NHL	R	24	1	> 17857000	374/0.44/1.22	Entecavir	NA	Alive, response[1]
5	81	M	NHL	CHOP-R	30	12	660570	737/1/1.37	No treatment due to spontaneous seroconversion	Anti-HBc (+)/anti-HBs (+) 22 IU/L	Alive, response[1]
6	78	M	Castleman's disease	P, R	12	1 (from last R cycle)	4228720	1536/15.8/1.03	Lamivudine	Anti HBc (+), anti- HBs (+) 189 IU/L	Non-liver related death
7	82	M	CLL	Chl, R, C	22	17 (from last R cycle)	> 17857000	3440/20.8/1.7	Tenofovir	HBsAg (+), anti-HBc (+), HBeAg (+)	Alive, response[1]
8	72	M	Temporal arteritis	P, MTX	6	0	> 17857000	308/0.63/1.03	Tenofovir	NA	Alive, response[1]
9	75	M	Dermatomyositis	P, MTX, AZA, Cyc	15	0	126992	657/1.47/0.86	Entecavir	Anti-HBc (+)/anti-HBe (+)	Alive, response[1]
10	53	M	Kidney transplantation	P, Cyc, Myc	48	0	> 17857000	28/0.96/0.98	Entecavir	HBsAg (+), anti-HBc (+), HBeAg (+)	Alive, response[1]
11	73	F	NHL	CHOP-R	9	4	127000	614/1.2/1.12	Lamivudine	Anti-HBc (+) anti-HBs (+) 183 IU/L	Liver related death
12	68	M	HL	ABVD, CHOP-R, DHAP, HSC transplantation, BEAM	28	16	> 17857000	49/1.1/1.01	Tenofovir	HBsAg (+), anti-HBc (+), HBeAg(+)	Alive, response[1]
13	77	M	Waldenstrom's macroglobulinemia	C, R	36	3 (from last R cycle)	> 17857000	2500/19/1.19	Tenofovir	Anti-HBc (+)/anti-HBs (+) 579 IU/mL	Alive, response[1]
14	64	F	RA	MTX, R	24	2 (from last R cycle)	> 17857000	72/1.02/0.96	Entecavir	NA	Alive, response[1]

[1]Clinical and biochemical response to antiviral treatment. HBV: Hepatitis B virus; HBc: Hepatitis B virus core antibody; HBsAg: Hepatitis B virus surface antigen; F: Female; M: Male; NHL: Non-Hodgkin lymphoma; CLL: Chronic lymphocytic leukemia; IMS: Immunosuppression; C: Cyclophosphamide; Flud: Fludarabin; R: Rituximab; CHOP-R: Cyclophosphamide Doxorubicin Vincristine Prednisone-Rednizolon; Chl: Chlorambucil; MTX: Methotrexate; AZA: Azathioprine; Cyc: Cyclosporine; Myc: Mycophenolate. NA: Not applicable.

of 10 patients (71.4%) was hematologic malignancy: six suffered from non-Hodgkin lymphoma, one from Hodgkin lymphoma, one from Castleman's disease, one from chronic lymphocytic leukemia and one from Waldenstrom's macroglobulinemia (Table 1). Regarding the remaining 4 patients, 3 were diagnosed with rheumatological diseases (one with temporal arteritis, one with dermatomyositis and one with rheumatoid arthritis). Finally, the last patient had received kidney transplantation for chronic renal failure due to diabetes mellitus (Table 1). He was anti-HBc (+)/anti-HBs (-)/HBsAg (-) before kidney transplantation and he had received a cadaveric kidney from an HBsAg (-)/anti-HBc (-) donor.

Eleven out of 14 patients (78.6%) had received rituximab (R) as part of the immunosuppressive schedule regimen (Table 1). The patient suffering from Hodgkin lymphoma (patient 12) had sequentially received diverse schemes of chemotherapy, including combination with R and finally autologous hemopoietic stem cell transplantation (HSCT). The median time from the initiation of immunosuppression till HBV reactivation was 18.5 (range 6-48) mo. The median time from last chemotherapy cycle till HBV reactivation for the 10 out of 11 patients who received R was 3 (range 2-17) mo. Patient 12 was diag-

nosed with HBV reactivation 16 mo after HSCT. The remaining 3 patients experienced reactivation while on immunosuppression containing corticosteroids (Table 1).

The viral load during the diagnosis of HBV reactivation was high in 50% of the patients (above 17857000 IU/mL). The median viral load in the sera of the remaining patients was 574252 IU/mL (range 12700-4228720 IU/mL). The median maximum ALT, aspartate aminotransferase, bilirubin and INR levels during reactivation was 635.5 IU/mL (range 28-3440 IU/mL), 339 IU/mL (range 24-2306 IU/mL), 1.47 mg/dL) (range 1-20.8 mg/dL) and 1.075 (range 0.86-1.7), respectively. However, all our patients were asymptomatic during reactivation and were diagnosed in routine laboratory testing.

All but one patients received antiviral treatment with nucleos(t)ide analogues immediately after the diagnosis of HBV reactivation (Table 1). Actually, the median time from the documentation of transaminase rise until treatment initiation was 15 (range 0-180) d. Patient 5 had already achieved seroconversion (disappearance of HBsAg and development of anti-HBs Abs) at diagnosis of HBV reactivation and for this reason he did not receive any treatment.

The HBV serological markers of 10 patients who were tested at the end of follow-up, are shown in Table 1. In more detail, 4 (40%) patients seroconverted to anti-HBs Abs after a median of 10.5 (range 3-60) mo. Three (30%) patients remained HBsAg (+)/HBeAg (+) after 24 mo the two and after 1-mo the third whereas the remaining 3 (30%) patients were anti-HBc (+)/ anti-HBe (+) at the end of follow-up (after 1, 7 and 26 mo, respectively).

Regarding the outcome, 3 patients (21.4%) deteriorated, manifesting ascites and hepatic encephalopathy and 2 (14.3%) of them died due to liver failure. The third died of a non-liver related cause, 60 mo after the diagnosis of HBV reactivation.

DISCUSSION

The natural course of chronic HBV infection is determined by the interplay between virus replication and the host's immune response[17]. In case of OBI, a long-term persistence in the nuclei of the hepatocytes of the HBV covalently-closed-circular DNA (cccDNA) supports its molecular basis[18]. In parallel, there is a strong suppression of the viral activity by the host's immune surveillance, which is likely to be the factor of outmost importance. However, this state of suppression of viral replication and gene expression may be discontinued by any kind of immunosuppression, leading to the development of a typical hepatitis B that often has a severe, even fulminant, clinical course.

To the best of our knowledge, this is one of the larger series of patients with OBI or resolved HBV infection who experienced HBV reactivation after receiving immunosuppression. The incidence of such a clinical adversity varies in the literature, depending on the population studied or the immunosuppressive regimen used. Among patients with OBI and malignancies the reported incidence fluctuates between 2.7%[6], for conventional regimens and 25%[19] when R is used. In patients with rheumatologic diseases the incidence of HBV reactivation in resolved infection is rather lower ranging from 2.2%[12] to 5.2%[11]. However, in the later study[11], when only patients treated with biological agents were taken into account, the incidence raised to 11.5%. Our series confirms two points: first the vast majority (71.4%) of the patients were diagnosed with hematologic malignancy and second approximately 80% of the patients had received R, sometime during their course.

R is a monoclonal antibody directed against the CD20 antigen expressed on the surface of normal and malignant B lymphocytes causing apoptosis. B cell plays a key role in the multiple immune responses against HBV: besides the production of neutralizing antibodies, it is an antigen presenting cell and enhances the cytotoxic response of CD8 T lymphocytes. Therefore, its destruction favors dramatically HBV replication. Our series is in accordance with the finding that R is a HBV reactivation risk factor even greater than corticosteroids, since in a series of patients with lymphoma treated with chemotherapy (CMT), the only significant difference between the reactivation group with resolved hepatitis and the group without reactivation was treatment with R[5]. In addition, three recent reports, including a meta-analysis of 184 case reports, have demonstrated that R containing regiments significantly increased the risk of HBV reactivation in OBI patients by five-fold compared to non-R regimens[5,19,20].

Since by definition CMT/immunosuppession decreases the ability of the immune system to respond, a period of time is necessary for immune reconstitution and subsequent attack on the liver, where a massive replication of HBV has taken place due to lack of "surveillance"[16]. For this reason, HBV reactivation often manifests in periods between cycles of CMT/immunosuppressive treatment or at the end of therapy after the recovery of the host immune system[21]. Therefore, in HBsAg carriers the time between last CMT cycle and HBV reactivation detection is variable: from 1-36 mo, usually ranging between 1 and 4 mo[22]. From the present study, it seems that the same is true also for resolved HBV infection, since the median time from last cycle to reactivation was 3 mo, although with wide variation from 2 to 17 mo. Therefore, it is crucial to closely follow-up patients with OBI after stopping immunosuppession, especially when they have received R, for at least 2 years. In addition, patients who are in continuous immunosuppression containing corticosteroids without R in the regimen, must be considered as vulnerable as those receiving cycles of CMT, since -especially after corticosteroid tapering- they can develop HBV reactivation at any time even after 4 years, as happened with patient 10 who had received kidney transplantation.

The clinical course of HBV reactivation usually begins with HBV replication, during which serum HBVDNA levels increase and in a second phase hepatitis occurs (2-3

wk after HBVDNA elevation) characterized by increase of transaminases and, occasionally appearance of symptoms such as fatigue, malaise and jaundice[22]. However, the reactivation can also present with fulminant hepatitis which intimates a poor prognosis. In the present cohort, 1/5 of the patients manifested fulminant liver failure and 14% died. In a recent meta-analysis of case reports and case series of R associated HBV reactivation in lymphoproliferative diseases[20], the fulminant liver failure rate and, as a consequence, the liver related mortality rate among the HBcAb (+)/HBsAg (-) cases was between 20% and 50%. The low mortality in our present case series could be attributed to the immediate initiation (within a median of 15 d of antiviral treatment after transaminase elevation. In many circumstances, this is not the case, since these patients are, frequently, not recognized as a reactivation high-risk group, leading to an underestimation of hepatitis due to HBV[23].

The vast majority of our patients received tenofovir or entecavir, drugs with high barrier to resistance. This can be justified by considering that current guidelines[15] recommend therapy with nucleoside analogue for at least 6-12 mo after discontinuation of chemotherapy and patients with hematological malignancies receive long term therapeutic regiments so they will probably demand long term use of antiviral treatment with the risk to develop treatment-resistant HBV variants if treated with lamivudine.

Male sex has been reported to be a significant factor associated with the risk of developing HBV reactivation in HBsAg (-) cancer patients on chemotherapy[24,25]. Interestingly, 12 out of 14 patients in the present study were male, which is in accordance with previous studies[19] on OBI patients. Male sex along with old age (median age at reactivation 74 years) could define two characteristics of OBI patients who reactivated after immunosuppression. It is unfortunate that blood samples were not available in our patients, for HBVDNA testing at baseline before the institution of immunosuppression, since in a recent study[26], HBVDNA testing had a 90% ability to forecast persistent HBsAg negativity in HBVDNA negative patients showing that highly sensitive serum HBVDNA testing had better performance than serological tests in predicting HBsAg reappearance.

In conclusion, patients with OBI who develop HBV reactivation after immunosuppression are more likely to have received R as a component of their treatment for their underlying disease, are more frequently older males and they can experience hepatitis due to HBV reactivation even 2 years after stopping the immunosuppressive therapy. Immediate start of antiviral treatment with potent antivirals after transaminase elevation and diagnosis of HBV reactivation with HBVDNA testing is of outmost importance in order to prevent deterioration and fulminant liver failure leading to lethal outcome. Two strategies could be adopted in order to prevent HBV reactivation in OBI patients: baseline and serial HBVDNA testing during and at least one year after the end of immunosuppressive treatment[23] or pre-emptive treatment with antivirals in all anti-HBc Abs (+) patients with or without resolved infection particularly when R is used. However, clinical evidence to date is not informative for determining optimal frequency and duration of such HBVDNA monitoring, whereas, concerning pre-emptive treatment, there are issues such as drug resistance and cost effectiveness, which also need to be addressed. Therefore, further studies to better define the characteristics of OBI patients who are "prone" to reactivation after receiving immunosuppression are needed, while in parallel establishment of new, precise guidelines on how to handle these patients seems to be extremely urgent considering the high mortality rate of a disease which can be effectively managed.

COMMENTS

Background

Patients with occult hepatitis B, defined by the presence of hepatitis B virus (HBV)-DNA in the liver tissue or also in serum of hepatitis B virus surface antigen (HBsAg) negative individuals, undergoing strong immunosuppression are potentially at risk of HBV reactivation, a common phenomenon in HBsAg-positive hematogical or oncological patients. HBV reactivation can be disastrous for the patient since it can lead to acute liver failure and death.

Research frontiers

The characteristics of these patients with occult HBV infection who experience HBV reactivation, when they receive immunosuppression as well as the features of the HBV reactivation itself need urgently to be defined. Until now few studies have accessed these topics mainly as case reports.

Innovations and breakthroughs

This is one of the larger series of patients with occult or resolved HBV infection who experienced HBV reactivation after receiving immunosuppression. The incidence of such a clinical adversity varies in the literature, depending on the population studied and, accordingly, on the immunosuppressive regimen used. This study confirms that the vast majority of these patients are being diagnosed with hematologic malignancy and the patients usually have received rituximab, sometime during their course.

Applications

Clinical evidence to date is not informative for determining optimal frequency and duration of HBV DNA monitoring during immunosuppressive treatment in patients with occult or resolved HBV infection. In addition, concerning pre-emptive treatment given in all these patients, there are issues such as drug resistance and cost effectiveness, which need to be addressed. Therefore, further studies to better define the characteristics of occult HBV infection patients who are "prone" to reactivation after receiving immunosuppression are needed.

Peer review

The excellent manuscript is of utmost importance and clinical relevance as it throws light on a frequently faced issue on handling of cases with occult HBV infection undergoing immunosupression.

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BRIEF ARTICLE

Hepatitis C virus genotypes in north eastern Algeria: A retrospective study

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Abstract

AIM: To determine the frequency of various hepatitis C virus (HCV) genotypes present in patients from north eastern Algeria.

METHODS: This is a retrospective cross-sectional study of 435 HCV infected patients from northeast Algeria, detected in the Sadelaoud laboratory and diagnosed between January 2010 and December 2012. The patients were diagnosed with HCV infection in their local hospitals and referred to be assessed for HCV genotype before the antiviral treatment. Demographic information (sex, age and address), genotype, subtype and viral load were retrieved from the patient medical records. The serum samples were tested by the type-specific genotyping assay.

RESULTS: The majority of the patients (82.5%) were from the central part of the examined region (P = 0.002). The mean age of the patients studied was 53.6 ± 11.5 years. HCV genotype 1 was the most frequent (88.7%), followed by genotypes 2 (8.5%), 4 (1.1%), 3 (0.9%) and 5 (0.2%). Genotype 6 was not detected in these patients. Mixed infection across the HCV subtypes was detected in twenty patients (4.6%). The genotype distribution was related to age and region. Genotype 1 was significantly less frequent in the ≥ 60 age group than in the younger age group (OR = 0.2; 95%CI: 0.1-0.5, P < 0.001). Furthermore, genotype 1 was more frequent in the central part of the examined region than elsewhere (P < 0.01).

CONCLUSION: The HCV genotype (type 1b was dominant) distribution in Algeria is different from those in other northern countries of Africa.

Key words: Hepatitis C virus; Prevalence; Genotype 1b; Viral load; Algeria

Core tip: Hepatitis C virus (HCV) infection is a common worldwide health problem; it is one of the major causes of chronic liver disease. HCV has at least six genotypes. The distribution of HCV genotypes varies greatly over the world. Genotype identification is clinically important to tailor the dosage and duration of treatment. The prevalence and HCV genotypes in Algeria are not known. In this study, we found that HCV genotype distribution in Algeria is different from the distribution detected in other northern countries of Africa.

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INTRODUCTION

Hepatitis C virus (HCV) infection is a major global public health issue. It is estimated that the global prevalence of HCV is approximately 2.8% (or 180 million people)[1] of the total population. HCV has a high viral heterogeneity. According to the nucleotide divergence, there are at least six genotypes, each of them containing a series of subtypes[2]. HCV genotypes have a striking geographical and epidemiological distribution and genotype identification is clinically important to tailor the dosage and duration of treatment because of different patterns of the treatment response and, consequently, distinct therapeutic approaches are required for each genotype[3]. In several areas of the world, HCV genotype 1 is reported as the most common infecting genotype among chronically infected patients. HCV genotypes 1, 2 and 3 appear to have a worldwide distribution and their relative prevalence varies from one geographical area to another[4]. HCV subtypes 1a and 1b are the most common genotypes in the United States[5] and Europe[6]. The predominant subtype reported from Japan is subtype 1b, responsible for up to 73% of cases of HCV infection[7,8]. HCV subtypes 2a and 2b are relatively common in North America, Europe and Japan and subtype 2c is found commonly in northern Italy[5,6]. HCV genotype 4 appears to be prevalent in north Africa and the Middle East[9,10] and genotypes 5 and 6 seem to be confined to South Africa and southeast Asia, respectively[11,12]. The north African data are based on information from only Egypt, Libya, Tunisia and Morocco. However, a published study on HCV genotype prevalence in Algeria[13-15] does not exist. Preliminary data by Benabdellah *et al*[16] reported that genotypes 2a/2c were predominant (47%) in 140 patients retrospectively evaluated between 2005 and 2011.

The aim of our study is to identify the prevalence of different HCV genotypes in north eastern Algeria and to assess the correlation between the HCV genotypes and demographic profile.

MATERIALS AND METHODS

We retrospectively evaluated 435 HCV infected patients examined between January 2010 and December 2012 in the Sadelaoud laboratory, a regional medical laboratory in the city of Batna and the only molecular biology laboratory in the eastern area of the country. The patients were diagnosed with the HCV infection in their local hospitals and referred to the Sadelaoud laboratory to be assessed for HCV genotype before antiviral treatment. We retrieved demographic information (sex, age and address), genotype, subtype and viral load from the patient medical records. The patients who were evaluated live in fifteen wilayas (provinces), the administrative regions which cover the eastern area of Algeria. These wilayas were classified into three regions for this study: the central part covering five wilayas (Batna, Khenchela, M'Sila, Oum El Bouaghi and Tebessa), the northern part (Annaba, Bordj Bouararidj, Constantine, Guelma, Jijel, Mila, Setif) and the southern part (Biskra, El Oued and Ouargla) (Figure 1).

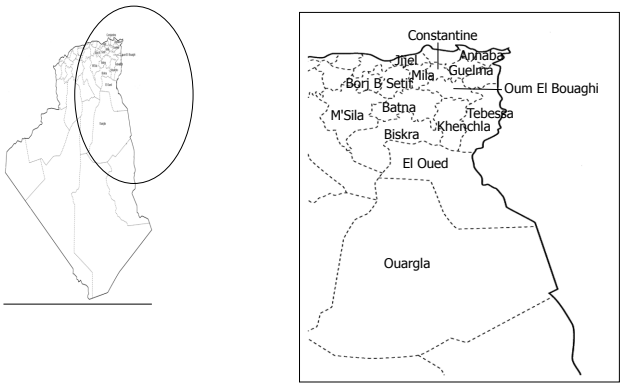

Figure 1 Hepatitis C virus genotype study coverage.

The HCV-RNA quantification was done by real time PCR (AmpliPrep/Cobas Taqman, Roche Molecular Systems, Branchburg, NJ, United States) with lower limit of detection of 15 IU/mL. Genotyping was done by INNO-LiPA HCV assay: Versant HCV genotype 2.0 assay (Siemens HealthCare Diagnostics, Tarrytown, NJ, United States). The genotype and HCV-RNA quantification were determined in a single laboratory, the Sadelaoud laboratory in Batna. The data about possible risk factors for HCV transmission were not available in the laboratory database.

Statistical analysis

Pearson's χ^2 and Fisher's exact tests were used to assess the differences in the patients' characteristics of the genotype, subtype, viral load, age, sex and region. Age was categorized into two groups: < 60 years and ⩾ 60 years and the genotype into two groups: "genotype 1" and "others" respectively. To assess the genotype distribution according to age and region, binomial logistic regression was performed with *P*: probability to be infected by genotype 1. The interaction of the covariate region on the association between the age and genotype was tested by the likelihood ratio test comparing the models with and without the interaction term. The interaction was significant if $P < 0.05$. The statistical analysis was done using the R version 2.15.1 statistical software.

RESULTS

Demographic features

The main demographic characteristics of the patients are shown in Table 1. The majority of the patients (82.5%, 359/435) were from the central part of the examined region ($P = 0.002$). The mean age of the patients studied was 53.6 ± 11.5 years (range 20 to 86 years) but over two thirds of the patients (70.6%, 307/435) were > 50 years old. There was a clear predominance of females (F/M ratio = 1.9). The female predominance was significant in all age groups ($P = 0.04$) except for the youngest in which we observed male predominance.

Table 1 Distribution of study population *n* (%)

Variables	Total	Sex		*P* value
		Male, 150 (34.5)	Female, 285 (65.5)	
Age (yr)	20-29	12 (8)	7 (2.5)	0.04
	30-39	13 (8.7)	20 (7)	
	40-49	28 (18.7)	48 (16.8)	
	50-59	50 (33.3)	128 (44.9)	
	60-69	33 (22)	63 (22.1)	
	> 70	14 (9.3)	19 (6.7)	
Region	Central	112 (74.66)	247 (86.66)	0.002
	North	31 (20.66)	26 (9.12)	
	South	7 (4.66)	12 (4.21)	

Table 2 Hepatitis C virus genotypes in eastern Algerian population

Genotype	*n* (%)	Subtype	*n* (%)
1	386 (88.7)	1a	6 (1.55)
		1a/1b	1 (0.26)
		1b	375 (97.2)
		Undefined subtypes	4 (1.0)
2	37 (8.5)	2a/2c	18 (48.6)
		2a	1 (2.7)
		2b	2 (5.4)
		Undefined subtypes	16 (43.2)
3	4 (0.9)	3a	4 (100)
4	5 (1.1)	4a	1 (20)
		4a/4c/4d	1 (20)
		Undefined subtypes	3 (60)
5	1 (0.2)	5a	1 (100)
Unclassified	2 (0.4)		2 (100)

Viral load

The viral load was assessed in all patients and the values were reported to the threshold of 600000 IU/mL: 276 patients (63.4%) had a viral load ⩾ 600000 IU/mL and 159 (36.6%) had a viral load < 600000 UI/mL.

HCV genotypes

Five genotypes (1 to 5) and ten subtypes of HCV were identified in the studied population (Table 2). HCV genotype 1 was the most prevalent (88.7%), followed by genotypes 2 (8.5%), 4 (1.1%), 3 (0.9%) and 5 (0.2%). Genotype 6 was not detected in these patients. The most prevalent subtype was subtype 1b (86.2% out of the total). Twenty patients (4.6%) had mixed infection across the HCV subtypes: eighteen within subtype 2a/2c, one within subtype 1a/1b and one case with subtype 4a/4c/4d. There were no patients with mixed genotype infection. Genotypes 1 and 2 were found in the majority of wilayas (fourteen and ten respectively) (Figure 2). Genotypes 3 and 5 were found in the wilayas of Setif and Constantine respectively, and genotype 2 was found in the wilayas of Batna, Oum El Bouaghi and Setif.

Relationship between genotype and demographic profile

The genotype distribution is related to the age and region (Table 3). Genotype 1 was more frequent than the other genotypes in all age groups and regions. Genotype 1 was significantly less frequent in the ⩾ 60 age group than in the younger age group (OR = 0.2; 95%CI: 0.1-0.5, $P < 0.001$) (Table 4). Furthermore, genotype 1 was more frequent in the central part than elsewhere ($P < 0.01$). We did not find a significant association between the HCV genotype and sex or viral load (Table 3).

Table 3 Hepatitis C virus genotypes according to age, sex, region and viral load *n* (%)

Risk factors	Genotype 1	Other genotypes	*P* value
Age (yr)			< 0.0001
< 60	284 (65.3)	22 (5.1)	
⩾ 60	102 (23.4)	27 (6.2)	
Sex			0.8
Male	132 (30.3)	18 (4.1)	
Female	254 (58.4)	31 (7.1)	
Region			< 0.01
Central	327 (75.2)	32 (7.4)	
North	43 (9.9)	14 (3.2)	
South	16 (3.7)	3 (0.7)	
Viral load (IU/mL)			0.2
< 600000	144 (33.1)	15 (3.4)	
⩾ 600000	241 (55.4)	33 (7.6)	

Table 4 Relationship between hepatitis C virus genotype and age

Age group (yr)	*n*	Genotype 1	Crude OR (95%CI)	Adjusted OR by region (95%CI)	Interaction Age × region
< 60	306	92.8%	0.30[b] (0.2-0.5)	0.20[b] (0.1-0.5)	No
⩾ 60	129	79.1%			

[b]$P < 0.001$ *vs* genotype 1.

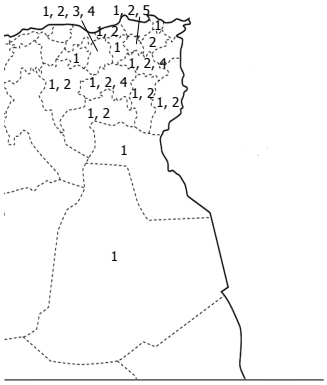

Figure 2 Hepatitis C virus genotype geographical pattern in eastern Algeria.

DISCUSSION

This is the first study to establish HCV genotype prevalence carried out on a large number of patients covering the north eastern geographical region of Algeria. Genotype 1b was a significantly predominant (86.2%) type. The result differs from what was reported in north western Algeria where the genotype 2a/2c was predominant (47%)[16]. In our study, genotype was not determined in two patients

(unclassified genotype). This could be due to the very low HCV viral load in those two patients. Also, 23 patients have an undefined subtype which may be due to the technical limits of our genotype determination tool.

The HCV genotype distribution is similar to those found in some neighboring countries; HCV genotype 1b is a dominant genotype in the studies conducted in Tunisia (84%) and Morocco (70.1%)[13,17-19]. However, the HCV genotype prevalence in our study differs from what has been reported in other countries of North Africa. In Libya, genotypes 1 and 4 are predominant among the patients chronically infected with HCV, 35.7% and 32.6% respectively[20,21], and in Egypt, genotype 4 is quasi-exclusive (91%) and genotype 1 never exceeds 10%[22,23]. Compared to our data, other Mediterranean countries, in particular France and Italy, report a lower prevalence of genotype 1 (57% and 62% respectively)[6].

We examined the distribution of HCV genotypes and the gender associated genotypes in this study. The results clearly show that there is no variation among HCV genotypes and gender as the different HCV genotypes were distributed with the same ratio in males and females. In contrast to our observation, HCV genotypes were not distributed with the same pattern detected in Algeria in males and females in Libya. In Libya, the prevalence of HCV genotype 1 was found to be significantly associated with males, while genotype 4 has frequently been found in females[18].

The distribution of HCV genotypes may vary due to the age of the population. In the United States and western European countries, HCV non-genotype 1 is increasingly prevalent in younger patients and this is attributed to risk exposure differences[6]. In our study, genotype 1 is associated with the age group younger than 60 years and is lower in the older age group. To the contrary, the HCV non-genotype 1 was higher among the patients over 60 years. This can be explained by the changing patterns of transmission of the infection related to the change of the health system in the country after its independence 50 years ago. Indeed, during colonization, the majority of the population lived in rural areas without hospitals; traditional medicine was widely used. After independence, the use of modern medicine and hospitalization was more frequent. The identified HCV genotypes showed regional differences in our study and the central part was significantly the most infected region by genotype 1. Also, there was no correlation between genotype distribution and viral load. The threshold of 600000 IU/mL was chosen because this threshold predicts sustained virological response in treated patients with genotype 1 HCV infection[24,25].

Our work is the first that evaluates the distribution of genotypes of HCV in north eastern Algeria. However, it has some limitations related to the retrospective design of the study and a selection bias is possible given the use of the data from a single laboratory, but in our case it is somewhat mitigated because the laboratory is the only one in the region.

In conclusion, we found that HCV 1b is a predominant genotype in eastern Algeria. Further studies are needed in different regions of the country to estimate the different epidemiology of the HCV genotypes.

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COMMENTS

Background

The hepatitis C virus (HCV) has at least six genotypes. Genotype identification is clinically important to tailor the dosage and duration of treatment. The distribution of HCV genotypes varies greatly over the world. However, there is no information from Algeria on this issue.

Research frontiers

The distribution of HCV genotypes varies greatly over the world. However, HCV genotype distribution in Algeria has not been known so far. As Algeria is close to African and European countries, the distribution of genotypes may be influenced by this geographical location.

Innovations and breakthroughs

This is a retrospective study to identify the prevalence of different HCV genotypes in Algeria and assess the correlation between the HCV genotype and demographic profile. It is the first study of its kind performed in the north eastern region of Algeria.

Applications

Genotype identification is clinically important to tailor the dosage and duration of treatment. The determination of HCV genotype distribution in Algeria can predict antiviral treatment needs and explain the possible risk factors for HCV transmission.

Peer review

The authors present very important information regarding the still open issue of HCV genotype distribution in Algeria. It is well written.

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CASE REPORT

An isolate alpha-fetoprotein producing gastric cancer liver metastasis emerged in a patient previously affected by radiation induced liver disease

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Abstract

We report a case of an isolated hepatic neoplasia which originated in a site of the liver previously affected by radiation induced liver disease (RILD) in a patient resected for gastric cancer and referred to us for high serum alpha-fetoprotein (AFP) levels. This case challenged us in distiguishing, even histologically, between primary liver cancer and AFP producing gastric cancer metastasis. Only a panel of immunohistochemical markers allowed the definitive diagnosis of liver metastasis of endodermal stem cell-derived and AFP producing gastric cancer. We discuss the criteria for a differential diagnosis, as well as the possible link between RILD and emergence of liver neoplasia.

Key words: Alpha-fetoprotein producing gastric cancer; Hepatoid adenocarcinoma; Liver metastasization; Radiation induced liver disease

Cardinale V, De Filippis G, Corsi A, La Penna A, Rossi M, Catalano C, Bianco P, De Santis A, Alvaro D. An isolate alpha-fetoprotein producing gastric cancer liver metastasis emerged in a patient previously affected by radiation induced liver disease. *World J Hepatol* 2013; 5(7): 398-403 Available from: URL: http://www.wjgnet.com/1948-5182/full/v5/i7/398.htm DOI: http://dx.doi.org/10.4254/wjh.v5.i7.398

INTRODUCTION

Several case reports of alpha-fetoprotein (AFP) producing gastric cancer have been described[1-5]. It represents 3.9%-11% of all the neoplasia of the stomach collected in a Japanese series. Other than AFP production, gastric cancer and hepatocarcinoma (HCC) can share common histology features, as demonstrated in the case of the hepatoid adenocarcinoma (HAC) of the stomach. As described by Terracciano *et al*[6], HAC is an extrahepatic tumor with morphological features similar to HCC, often producing large amounts of AFP. The findings from Terracciano *et al*[6] suggest that HAC arises from gastric adenocarcinoma with an intestinal phenotype as a result of the further progression of the carcinogen process. The stomach is the organ where the tumor was most fre-

quently described and is associated with a poor prognosis for vascular invasion, regional lymphadenopathy and frequent liver metastases[1-5]. Terracciano *et al*[6] showed positivity in virtually all HACs for AFP, cytokeratin-8 (CK-8), CK-18 and carcinoembryonic antigen, markers underlining its hepatoid nature, the contemporary positive staining for CK-19 and CK-20, and the frequent negativity of Hep Par1 in both primary tumors and their metastases. Furthermore, HAC differs from combined hepatocellular cholangiocarcinoma, because it is negative for CK-7[6]. Because HAC liver metastases and HCC cannot be differentiated on the basis of morphology alone, the described pattern of phenotypical markers is necessary for differential diagnosis.

Radiation induced liver disease (RILD), previously referred to as "radiation hepatitis", can affect patients 4 to 8 wk after liver exposure to radiation[7-12]. RILD is a veno-occlusive disorder caused by a direct radiation-induced injury of the liver endothelium[10-12]. This condition can affect 6% to 66% of patients exposed to an excess of 30 to 35 Gy of radiation, depending on the volume of irradiated liver and hepatic functional reserve[10,12]. The typical clinical presentation is a triad of ascites, hepatomegaly and elevated liver enzymes[9]. Most patients recover completely after 3 to 5 mo but a minority will progress to a chronic phase, with hepatic fibrosis and liver failure; rare cases of fulminant liver failure have been described[10,12]. The diagnosis essentially emerges from the findings of the typical imaging features on contrast-enhanced computed tomography (CT) and/or magnetic resonance imaging (MRI), affecting the liver in the irradiated field: "straight-border" sign[8], demarcated areas of hypo or hyper attenuation in a non anatomical distribution (contrasting with vascular lesions)[8], and alteration of the contrast uptake on CT[13].

We describe a case AFP producing liver metastatic gastric cancer in a patient affected by RILD and the implications in terms of diagnostic challenges and pathogenesis.

CASE REPORT

A 52-year-old male, heavy smoker (40 cigarettes per day for 30 years), 1.5 years before our first examination, underwent gastrectomy for gastric cancer of the angulus, stage Ⅱ (pT2b, pN1, pMx) of the TNM Classification of Malignant Tumors[14], grade 3 (G3) according to the American Joint Commission on Cancer[15]. Blood analysis and liver imaging [contrast-enhanced CT, ultrasound sonography (US)] performed during hospital stay for gastric surgery demonstrated the absence of risk factors for liver disease and a normal liver structure and function. Preoperative serum tumor markers were not evaluated. Three months after surgery, he underwent adjuvant therapy: 5-fluoruracil (5-FU) *ev* infusion 225 mg/mq per die (35 d) plus 45 Gy irradiation on the epigastric region. During follow-up 6 mo after surgery, a diffuse alteration of the contrast medium uptake affecting the left hepatic lobe was evidenced by a control CT performed without an emerging clinical indication. CT showed band-like hypo attenuation in the liver with parenchymal swelling corresponding to the radiation field. Interestingly, the CT showed a thin layer of ascites. On MRI, the diseased area appeared like a rectangular shape affecting the left liver lobe and the hilar region, which resulted in hypo intense on T1-weighted images, iso and hyper intense on T2-weighted images in the pre-contrast phase, and hypo intense in the post-contrast images (Figure 1). No alteration of liver serum enzymes was detected. The contrast enhanced imaging follow-up, through both CT and MRI, showed a complete recovery of the liver parenchyma 10 mo after irradiation, suggesting the diagnosis of RILD. The patient did not show any alteration of serological liver parameters, except for high AFP serum levels [13 fold above normal values (NV)], whose first evaluation dated back to a period of the post surgical follow-up when the liver lesion was disappearing. After upper and lower endoscopy examinations which resulted negative for neoplasia, the patient was referred to our gastroenterology unit in May 2008 due to a further increase of the AFP level (21 fold above NV). In the same time period, other tumor markers such as carcinoembryonic antigen and carbohydrate antigen 19-9 were normal. At this time, HCV-Ab and hepatitis B surface antigen were negative and transaminases, indexes of cholestasis and prothidosynthesis were in the normal range. No evidence of metabolic disease or iron accumulation emerged. The patient had an average intake of three standard alcohol drinks per day (12 g of ethanol per standard alcohol drink) and was treated with ramipril for mild essential hypertension. Finally, no instrumental signs of portal hypertension by US were found. On physical examination, the liver was palpable (2 cm under the costal arch in inspiration), with parenchymatous consistency and a smooth surface, the spleen was not palpable, no peripheral lymphadenopathies were discovered and the testes were normal. Thus, the patient underwent an abdominal and testis US, total body dynamic contrast-enhanced CT and upper endos-

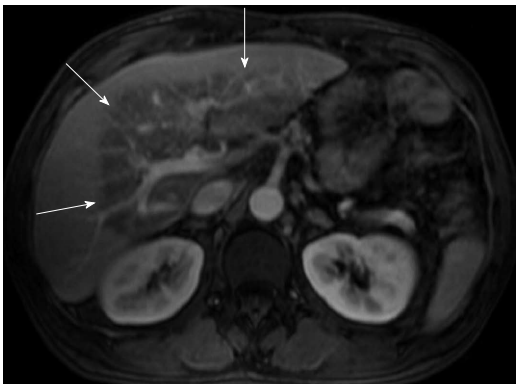

Figure 1 Spoiled gradient recalled-echo and fat-suppressed T1-weighted magnetic resonance imaging after *iv* gadolinium administration. The venous phase on the axial portal plane shows, at the level of the left hepatic lobe and of the liver hilum region, a triangular hypo intense area (arrows) with hyper intense small areas within; vascular structures are preserved (May 2007).

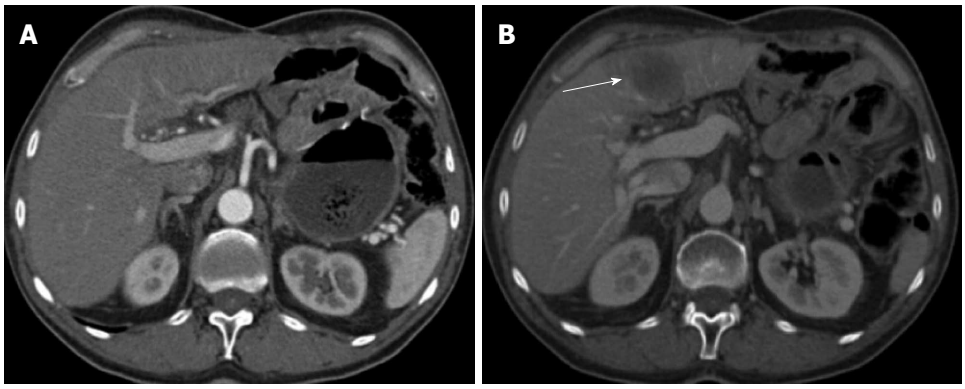

Figure 2 Axial computed tomography scan after *iv* iodinated contrast medium administration in the arterial hepatic phase (A) and portal phase (B). A: The computed tomography study does not show focal lesions in the liver parenchyma (Jul 2008); B: A focal nodular hypo attenuated lesion is present affecting the left hepatic lobe (arrow); it presents with regular margins and appears mildly vascularised (Nov 2008).

copy with gastric mucosa biopsies. All these examinations failed to detect alterations, except for a 1.5 cm lymph node at the site of the surgical anastomosis (Figure 2A). As a consequence of a dramatic increase of AFP serum levels (200-fold the NV) in September 2008, an US examination detected a 3 cm × 2 cm, hypoechogenic lesion of the left hepatic lobe which was biopsied. The total body dynamic contrast-enhanced CT confirmed a single 4 cm hypo vascular lesion affecting the left hepatic lobe, in the same region previously affected by RILD (Figures 1 and 2). The histology showed an extensively necrotic, poorly differentiated epithelial tumor (Figure 3A). The neoplastic cells were immunoreactive for MOC-31 (cell surface glycoprotein), CK-8 and CK-18 (Figure 3B and C) and negative for CK-7, CK-20 and Hep Par 1. Sparse cells were immunoreactive for AFP as well (Figure 3D). Since the upper endoscopic examination excluded pathology of the residual stomach mucosa, a presumptive diagnosis of a primary poorly differentiated liver tumor originated at the site affected by RILD was taken into consideration. However, a positron emission tomography examination showed 2 areas of increased metabolic activity localized in correspondence to a parasternal lymph node and in the left hepatic lobe. Those alterations were referred to as metastatic lesions. Other areas with a less pronounced metabolic activity were described as affecting the epigastric region and the mesenteric-pancreatic region. Therefore, the primitive gastric cancer (Figure 3E) was re-evaluated by immunohistochemistry. The analysis revealed diffuse positivity of the neoplastic cells for MOC-31, CK-8, CK-18 and AFP (Figure 3F-H), focal positivity for Hep Par-1 and negative for CK-7 and CK-20. The comparative histology analysis of the liver lesion and the gastric cancer allowed a definitive conclusion of liver metastasis of an AFP-producing gastric cancer. The patient was enrolled to receive systemic chemotherapy for gastric cancer metastases. The patient deteriorated from progressive hepatic metastases and expired 12 mo after the detection of the first liver metastatic lesion. No autopsy was performed.

DISCUSSION

This case report engaged us in a differential diagnosis between HCC and AFP-producing gastric cancer metastasis. The final diagnosis of the liver lesion was achieved only by immunohistochemistry, where MOC-31 (cell surface glycoprotein), CK-8 and CK-18 positivity, focal positivity for AFP and negativity for CK-7, CK-20 of both liver lesion and gastric cancer allowed the definitive conclusion of liver metastasis of AFP-producing, and likely endodermal stem cell-derived, gastric cancer. Moreover, this phenotype is in accordance with the features of hepatoid adenocarcinoma of the stomach, as described by Terracciano *et al*[6]. As a differential diagnosis, we hypothesized a case of primitive liver cancer originating on RILD. This hypothesis was sustained by the potential mutagen effect of radiation[16] and chemotherapy, other than the absence of other metastatic lesions and the lack of local gastric recurrence. In contrast, the immunohistochemical analyses and the comparison with the primitive gastric cancer allowed the definitive conclusion of liver metastasis. Therefore, the message for clinicians from this case report could be that, in the presence of high AFP serum levels and history of gastric cancer, the immunohistochemical characterization of primitive cancer and liver lesion is absolutely necessary for a definitive diagnosis. Moreover, in our opinion, the accurate evaluation of a pre-existing chronic liver disease[17] is of relevance because its absence, as in our case, decreases the probability of primitive liver cancer, reinforcing the accuracy of the diagnostic process. Indeed, theoretically primitive liver cancer occurrence could be increased by eventual radiation administration during the activation of resident stem cell compartment occurring in chronic liver diseases[17-24]. The second point in the discussion is the relationship between RILD and the emergence of liver metastases. In our patient, the diagnosis of RILD was based on strictly accepted criteria, including a contrast-enhanced CT and MRI that allowed us to follow the evolution of the characteristic liver lesion until its resolution[7-9]. Moreover, the dose of radiation was compatible with RILD occurrence, similar to the timing of a RILD occurrence after radiation exposure. As RILD clinical presentation depends on the volume of irradiated liver and hepatic functional reserve, a localized exposure of the liver parenchyma in a patient with a good hepatic functional reserve could lead to a RILD without elevated liver enzymes or massive ascites, as in our patient. In addition, we performed all necessary clinical, biochemical and imaging procedures to exclude underlying liver diseases.

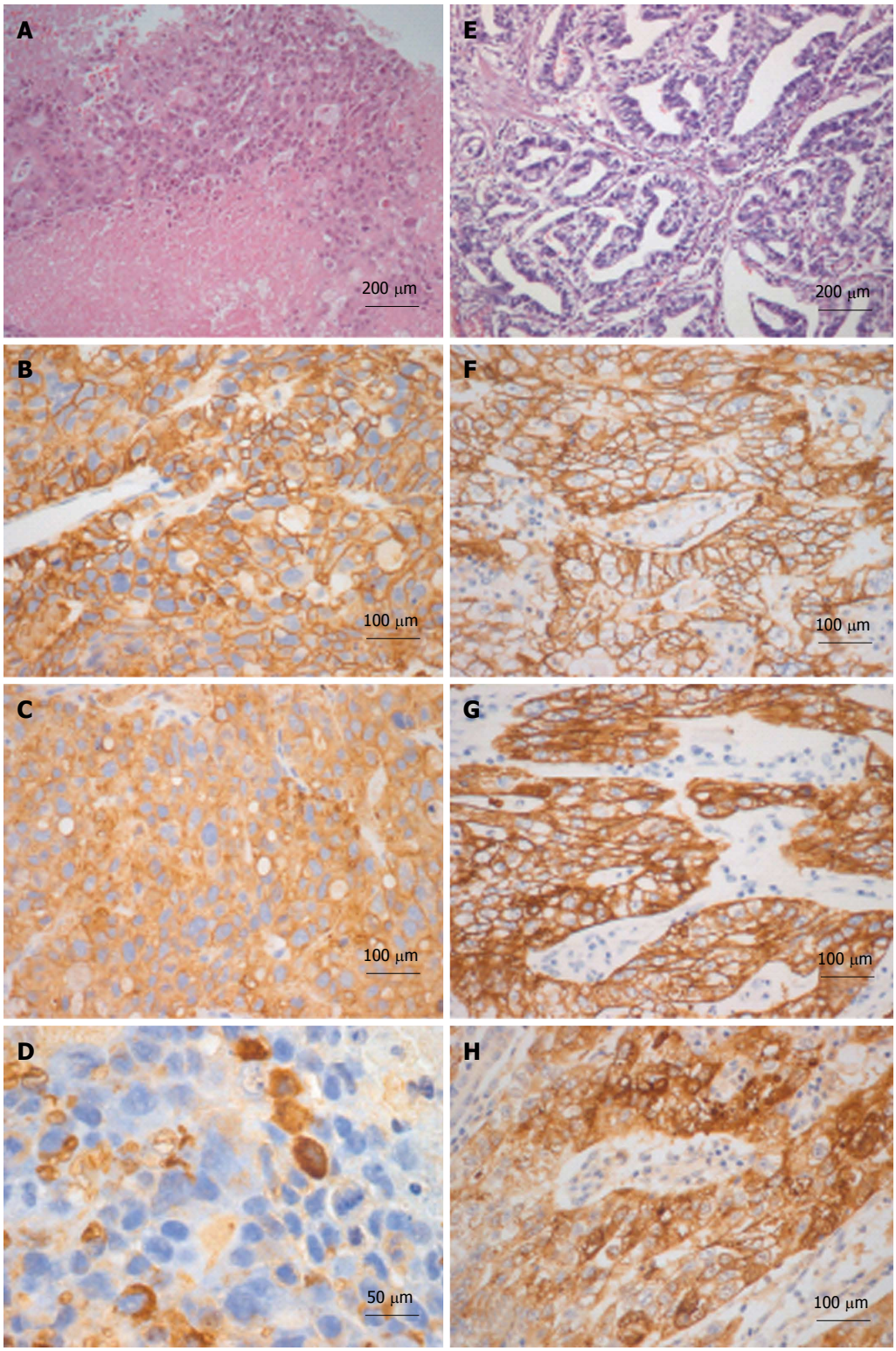

Figure 3 Histological-immunohistochemical characterizations of liver and gastric tumors are illustrated in A-D and E-H respectively. The liver tumor is extensively necrotic (A) and neoplastic cells are immunoreactive for MOC-31 (B) and cytokeratin (CK)-18 (C). Sparse cells are immunoreactive for alpha-fetoprotein (AFP) as well (D). The gastric adenocarcinoma (E) is immunoreactive for MOC-31 (F), CK-18 (G) and AFP (H) as well. A and E: Hematoxylin and eosin.

Whether AFP-producing gastric cancer is associated with frequent liver metastases[1-5], no cases of gastric cancer liver metastases emerging in a site of the liver previously affected by RILD were described. However, we were encouraged to describe the case because, in our opinion, the localization of metastasis in the site of the liver involved by RILD could be not casual but could involve specific pathogenetic mechanisms. In fact, several clinical and experimental evidences suggested an increased incidence of metastases after radiation doses that were insufficient

to control the primary tumor[25-28]. Furthermore, *in vivo* studies in mice demonstrated an increased susceptibility of the lung and the liver irradiated prior to *i.v.* administration of tumor cells, to be affected by cancer cell engraftment[29-31]. Radiation could favor metastases seeding since vascular damage facilitates tumor cell intravasation[29] and because cell death caused by radiation may release soluble factors, promoting the engraftment and growth of circulating cancer cells[32]. A specific role could be suggested for vascular endothelial growth factor (VEGF) generated in the site of RILD-induced vascular and cell damage. In fact, obstruction of the hepatic microcirculation, as occurs during RILD, leads to an increased hepatic VEGF expression[33]. This growth factor might induce proliferation of previously dormant liver microtumors[34] or may exert chemo-attractant function on VEGFR (VEGF receptors) expressing circulating cells, leading to enhanced metastases seeding[35,36]. Interestingly, gastric carcinoma cells express different types of VEGF and relative receptors, including VEGF-C and VEGFR-3, which may play a role in the seeding and growth of cells in the site where enhanced VEGF production occurs[37]; in our case, the left liver lobe affected by RILD. In conclusion, it could be rational to consider that RILD increased the probability of gastric cancer metastases seeding due to the occurrence of vascular damage facilitating the tumor cells intravasation and due to the increased production of chemo-attractant molecules for malignant circulating cells, such as VEGF.

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CASE REPORT

A rare case of hyaline-type Castleman disease in the liver

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Key words: Castleman disease; Hyaline type; Liver tumor; Hepatectomy; Positron emission tomography

Core tip: We report a very rare case of hyaline-type Castleman disease in the liver. Castleman disease can occur wherever lymphoid tissue is found, although it rarely appears in the abdominal cavity, and is especially rare in the liver. The patient was suspected of having a malignant liver tumor because of positron emission tomography findings. No findings from diagnostic imaging specific to Castleman disease are known and it is, therefore, difficult to make a predictive diagnosis.

Miyoshi H, Mimura S, Nomura T, Tani J, Morishita A, Kobara H, Mori H, Yoneyama H, Deguchi A, Himoto T, Yamamoto N, Okano K, Suzuki Y, Masaki T. A rare case of hyaline-type Castleman disease in the liver. *World J Hepatol* 2013; 5(7): 404-408 Available from: URL: http://www.wjgnet.com/1948-5182/full/v5/i7/404.htm DOI: http://dx.doi.org/10.4254/wjh.v5.i7.404

Abstract

Castleman disease often develops in the neck, mediastinum and pulmonary hilum. Its onset in the peritoneal cavity is very rare. The patient, a woman in her 70s, was referred to our department for a detailed examination of an abdominal mass. On abdominal ultrasonography, computed tomography scan, magnetic resonance imaging and positron emission tomography, a mass approximately 15 mm in diameter was noted in the hepatic S6. We attempted radical treatment and conducted a laparoscope-assisted right lobectomy. On the basis of histopathological findings, the patient was diagnosed as having hyaline type Castleman disease in the liver, a very rare condition.

INTRODUCTION

Castleman disease, a lymphoproliferative disease with good prognosis that was first reported in 1954, presents with localized lymph node swelling[1]. Keller *et al*[2] divided this disease histopathologically into the hyaline type (characterized by hyperplasia of the hyalinated lymph follicles and vascularity accompanied by proliferation of vascular endothelial cell) and the plasma cell type (characterized by intense infiltration of plasma cells into the interfollicular tissue). It is now classified as an unexplained lymphoproliferative disease. Approximately 90% of cases are the hyaline vascular type and the other 10% are the plasma cell type[3]. The condition is known to develop in any age group regardless of gender. The hyaline type, which accounts for approximately 90% of all patients with Castleman disease, often develops in the neck, medi-

astinum, and pulmonary hilum. Its onset in the peritoneal cavity is very rare. Here we describe a very rare case of hyaline type Castleman disease in the liver.

CASE REPORT

The patient, a woman in her 70s, was referred to our department for a detailed examination of an abdominal mass. She had no noteworthy major complaint or disease history. Her mother had a history of colorectal cancer treatment and her husband had received liver cancer treatment. The patient developed epigastric pain in November 2011 and consulted a nearby clinic. At that time, an abdominal ultrasonography and computed tomography (CT) scan revealed a mass in the liver. She was then referred to our hospital.

Physical examination at our hospital revealed that the patient was 142 cm tall, weighed 47 kg, had a blood pressure of 143/63 mmHg, a heart rate of 71/min (regular), and a body temperature of 36.2 ℃. No superficial lymph nodes were palpable. Chest auscultation revealed no abnormalities. The abdomen was flat, soft, and not tender. No noteworthy neurological abnormalities were found.

No noteworthy abnormalities were detected by blood tests. Tumor markers (carcinoembryonic antigen, carbohydrate antigen 19-9, alpha-fetoprotein, and protein induced by vitamin K absense) were all within normal ranges.

On abdominal ultrasonography, a hypoechoic mass approximately 15 mm in diameter was noted in the hepatic S6. Radiography using perflubutane as a contrast agent revealed contrast enhancement during the vascular phase and a defect during the late vascular phase.

A plain CT scan of the abdomen revealed a well-demarcated low absorption area dimly visible in the S6. On dynamic CT scan, a thin membrane-like contrast enhancement was noted in the periphery during the arterial phase although no evident contrast enhancement was seen inside the mass. A mass with slightly low internal absorption was seen during the portal phase. A mass with slightly higher internal absorption compared to normal hepatic parenchyma was noted during the equilibrium phase.

On abdominal magnetic resonance imaging (MRI), a mass approximately 15 mm in diameter was visible in the hepatic S6 as a low signal intensity area on T1-weighted images and as a high signal intensity area on T2-weighted images. MRI using EOB Primovist disclosed a dimly contrast-enhanced mass during the arterial phase and a mass depicted as a low signal intensity area from the portal phase onward.

Positron emission tomography (PET) revealed enhanced accumulation with a standardized uptake value (SUV) of 6.1 noted in hepatic S6 (Figure 1).

No noteworthy abnormalities were seen in upper/lower endoscopy.

On the basis of these test results, the patient was suspected of having a malignant liver tumor (similar to a poorly differentiated hepatocellular carcinoma or cholangiocellular carcinoma). Because her hepatic reserves were favorable, we attempted radical treatment and conducted a laparoscope-assisted right lobectomy.

Finding with resected specimens

A white tumorous lesion approximately 20 mm in diameter and clearly distinguishable from surrounding tissue was noted.

Histopathological findings

Lymph follicle hyperplasia was noted in the affected liver tissue. Some follicles showed signs of vascular invasion, hyperplasia of the mantle layer, and the presence of multiple germ centers. Hyalinized interstitium was seen between follicles (Figure 2). There was no marked proliferation of lymphocyte-like atypical cells. Upon immunohistochemical staining, CD3, CD5, CD20, CD79a, CD10, CD21, CD23, and bcl-2 were all negative. Epstein-Barr virus-encoded RNA was also negative. On the basis of these findings, the patient was diagnosed as having hyaline-type Castleman disease in the liver, a very rare condition. This case was treated using surgical resection only. At the present time, the patient is under follow-up without any further treatment and the disease has shown no signs of recurrence.

DISCUSSION

Castleman disease, a lymphoproliferative disease with good prognosis but without an identified cause, presents with localized lymph node swelling. It was first reported by Castleman *et al*[1]. Pathologically, it can be divided into hyaline and plasma cell types. Clinically, it is classified into unicentric and multicentric types. The development manner and treatment methods vary by type[2,4-7]. The sites frequently affected by this disease are reported to be the mediastinum (65%), neck (16%), abdomen (12%), and axilla (3%)[8]. However, Jang *et al*[4] reported 10 rare cases of Castleman disease that developed in the hepatic hilum. In many of these cases, the disease was of the hyaline type located in the vicinity of the liver. The development of this disease inside the liver, as in the present case, may be viewed as very rare.

Clinically, cases of this disease with a single lesion usually have good prognosis, while cases with multiple lesions tend to have poor prognosis. On the basis of these characteristics, cases of this disease with a single lesion are classified as unicentric Castleman disease and cases with multiple lesions as multicentric Castleman disease (MCD). Most cases of MCD are pathologically rated as the plasma cell type[9]. In terms of clinical symptoms, lymph node swelling is often confined to particular regions in cases of hyaline-type Castleman disease, and symptoms other than those related to compression are rare for this type. On the other hand, the plasma cell type often shows signs of chronic inflammation such as fever, arthralgia, elevated erythrocyte sedimentation,

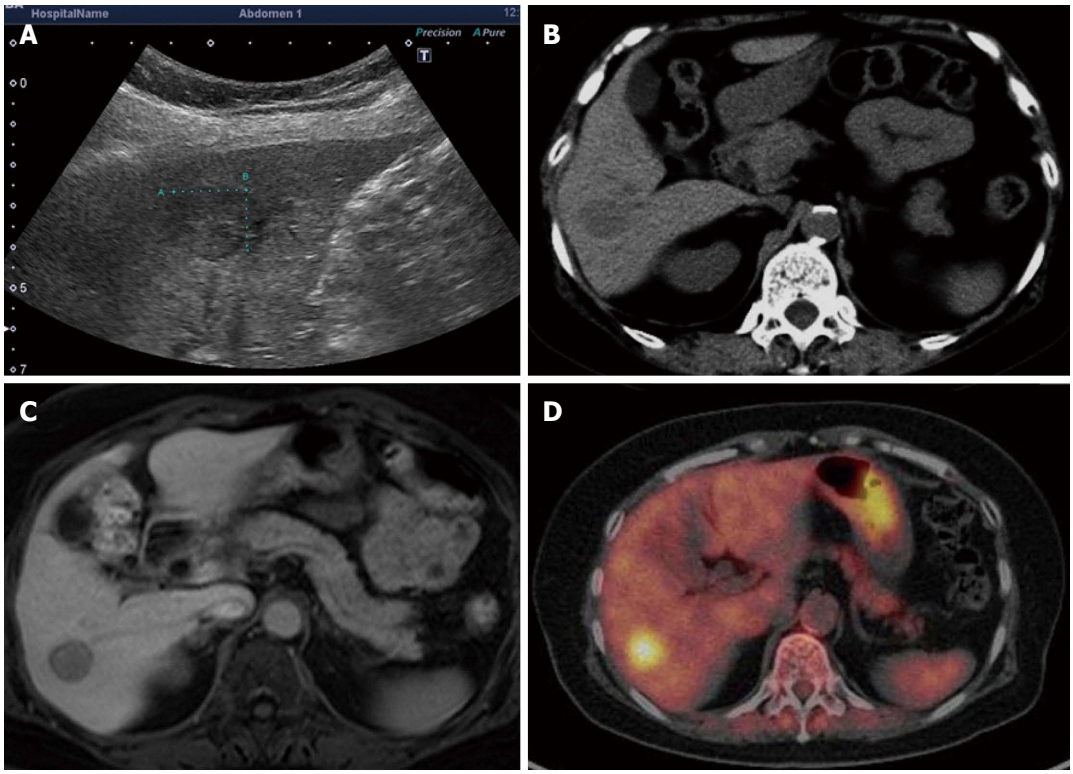

Figure 1 A mass approximately 15 mm in diameter was noted in the hepatic S6. A: Ultrasonography; B: Computed tomography scan; C: Magnetic resonance imaging; D: Positron emission tomography.

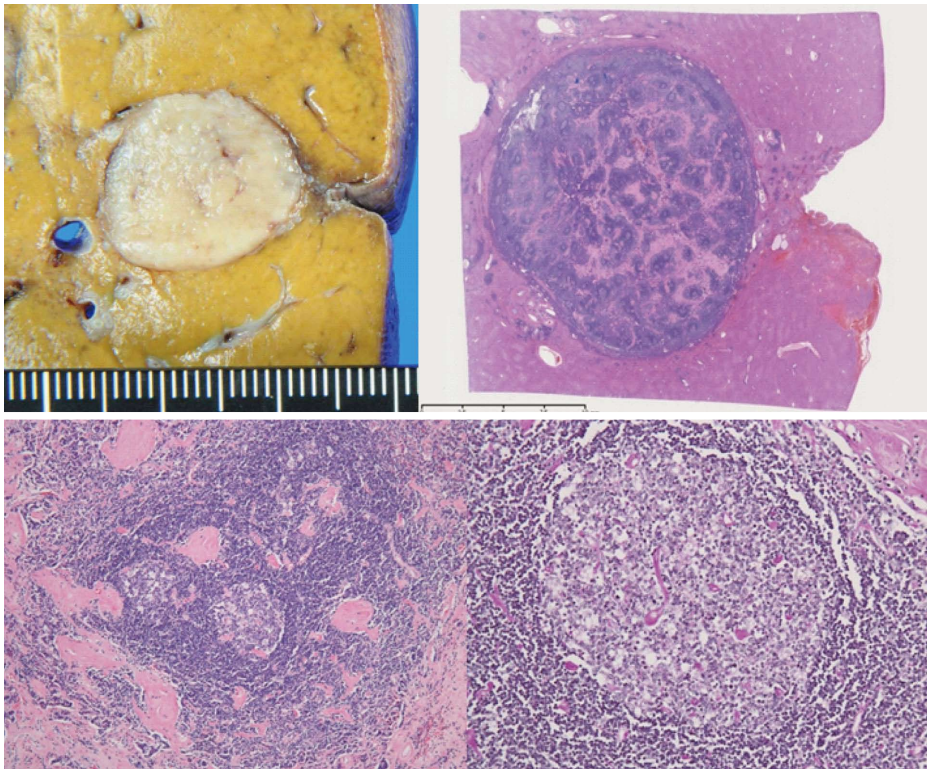

Figure 2 Lymph follicle hyperplasia was noted in the affected liver tissue. Some follicles showed signs of vascular invasion, hyperplasia of the mantle layer, and the presence of multiple germ centers. Hyalinized interstitium was seen between follicles.

weight loss, and systemic lymph node swelling[2]. These symptoms of the plasma cell type are sometimes accompanied by symptoms of Croe-Fukae syndrome (POEMS syndrome) such as polyneuritis, organ swelling, endocri-

nological abnormalities, M protein, and bone-sclerosing lesions[8,10].

No finding from diagnostic imaging specific to Castleman disease is known. When examined by ultrasonography, the affected area is often depicted as a well-demarcated hypoechoic mass with a homogeneous inside. On CT scan, a mass with high contrast enhancement, occasionally accompanied by calcification, is visible. When examined by MRI, this disease is often seen as a low signal intensity area on T1-weighted images and a high signal intensity area on T2-weighted images[11]. In fludeoxyglucose (FDG)-PET studies of Castleman disease sporadic moderate accumulation was noted in the mass[12,13]. Park *et al*[12] summarized the data from 10 cases, reporting that the mean SUV was 4.9 (range, 3.2-8.9). They added that this SUV is apparently lower than that known for high malignancy non-Hodgkin's lymphoma and close to that for low malignancy non-Hodgkin's lymphoma[12]. There are reports that FDG-PET gives positive findings in cases with non-malignant diseases such as sarcoidosis, tuberculoma, liver abscess, and chronic pancreatitis[14], indicating the need to bear in mind that patients with these inflammatory or granulomatous diseases can also be rated positive by FDG-PET.

Other conditions requiring distinction from this disease include liposarcoma, leiomyosarcoma, fibrohistiocytoma, gastrointestinal stromal tumor, and lymphoma. These diseases are poor in specific characteristics revealed by diagnostic imaging, making it difficult to make a preoperative diagnosis of Castleman disease. A preoperative tumor biopsy is not recommended for reasons such as an inability to collect a sufficient amount of tissue and the possibility to induce tumor dissemination by biopsy. In the present case, the FDG-PET finding of accumulation in the liver suggested a malignant tumor (poorly differentiated hepatocellular carcinoma, cholangiocellular carcinoma, metastatic liver cancer, or the like). We judged that this patient had a malignant tumor of hepatic origin for the following reasons: (1) absence of signs of malignancy revealed by upper/lower gastrointestinal endoscopy; and (2) absence of organs other than the liver showing abnormal accumulation during FDG-PET[8,15].

We attempted radical treatment by surgical resection in this case because of favorable hepatic reserves. However, there is no established method for treating Castleman disease[4]. It has been reported that in cases of hyaline-type Castleman disease with a single lesion, radical treatment with surgical resection alone is likely to be achieved and the prognosis is good. Although reports of the use of radiotherapy for cases not indicated for surgery have been published, the efficacy of radiotherapy remains controversial[5-7,16]. For plasma cell-type cases, treatment is sometimes attempted using cyclophosphamide, vincristine, doxorubicin, and prednisone or using dexamethasone, which resembles the therapy used for non-Hodgkin's disease[6,7]. In recent years, interleukin (IL)-6 has been suggested to be involved in the pathophysiology of Castleman disease, and cases responding well to anti-IL-6 therapy have also been reported[17].

It has been reported that the hyaline vascular type of disease often undergoes a gradual increase in lesion size over the course of several years or more after disease onset[3]. In the present case, surgical resection was used for both diagnosis and treatment since malignancy was not eliminated by the FDG-PET findings and because biopsy involved the risk of tumor dissemination. We believe that this strategy was rational for this case. Castleman disease is an unexplained lymphoproliferative disease that often develops in the mediastinum. As presented in this paper, we recently encountered a very rare case of this disease, hyaline type Castleman disease that developed inside the liver.

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

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Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
Biology: *H. pylori*, *E coli*, *etc.*

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