

B-Raf protein immunoexpression in hepatocellular carcinoma due to hepatitis C virus related cirrhosis

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ABSTRACT – Background – Hepatocarcinogenesis is a multistep process that lead to genetic changes in hepatocytes resulting in neoplasia. However, the mechanisms of malignant transformation seem to differ widely. To know carcinogenesis mechanisms is essential to develop new treatment and prevention methods. **Objective** – The aim of this study is to analyze B-Raf protein immunoexpression in explants with hepatocellular carcinoma (HCC) related to hepatitis C (HCV), in adjacent cirrhotic tissue and in normal livers. We also associated the immunoexpression with known HCC related histopathological prognostic features. **Methods** – Livers from 35 patients with HCV related cirrhosis and HCC that underwent liver transplantation or hepatectomy at Clinical Hospital – UFMG and 25 normal livers from necropsy archives were studied. Tumors were classified according to: tumor size, vascular invasion and differentiation grade. B-Raf protein expression was determined by immunohistochemistry. **Results** – B-Raf was strongly expressed in the HCV cirrhotic parenchyma cytoplasm of 17.1% cases and in 62.9% of HCC samples. Strong B-Raf protein staining was associated with tumor tissue ($P < 0.0001$; OR = 8.18 (2.62–26.63)). All normal livers showed weak or negative expression for B-Raf. There was no significant association among B-Raf scores and tumor differentiation grade ($P = 0.9485$), tumor size ($P = 0.4427$) or with vascular invasion ($P = 0.2666$). **Conclusion** – We found B-Raf protein immunostaining difference in normal livers, in the areas of HCV cirrhosis and in the hepatocarcinoma. We did not find association between B-Raf expression and histopathological markers of tumor progression. Our data suggests that B-Raf may play an important role in initial HCC carcinogenesis. Larger studies are needed to validate these observations.

Keywords – Hepatocellular carcinoma; B-Raf; hepatitis C virus.

INTRODUCTION

Primary liver cancer is the sixth most common cancer worldwide and the fourth main cause of death from cancer⁽¹⁾. Hepatocellular carcinoma (HCC) accounts for 85 to 90% of primary liver cancers⁽²⁾. The prominent agents associated with HCC include chronic hepatitis B and hepatitis C virus infection (HCV), chronic alcohol consumption, dietary exposure to aflatoxin-B1 and virtually all cirrhosis-inducing diseases⁽³⁾. In face of a still poorly understood etiopathogenesis, signaling pathways related to hepatocarcinogenesis have been the subject of constant studies⁽⁴⁾. Multiple signaling pathways that regulate cell proliferation, angiogenesis and vascular invasion may be altered in HCC. Among them, we can highlight those with the participation of BRAF gene.

BRAF is a proto-oncogene that encodes a serine/threonine kinase that transduces regulatory signals through Ras/Raf/MEK/ERK cascade⁽⁵⁾. This pathway mediates cellular response to growth signals. Somatic mutations of BRAF provide an alternative mode of aberrant activation of the MAPK signaling pathway that is implicated in many human cancers⁽⁶⁾. Up regulation of this signaling pathway has been well documented in HCC and correlates with advanced stage⁽⁷⁾.

HCV infection is the most frequent risk factor of HCC in Latin

America, representing 48% of the cases⁽⁸⁾. HCV contributions to hepatocarcinogenesis are supposed to be related with the viral proteins, such as core, NS3 and NS5A proteins^(9,10). It is believed that simultaneous evaluation of multiple genes and regulatory pathways in HCC should help to identify causative factors, markers for early detection and prognosis prediction, as well as new therapeutic approaches⁽¹¹⁾. As BRAF has been associated with hepatocarcinogenesis⁽¹²⁾, the aim of this study was to analyze the immune expression of its encoded protein, B-Raf, in explants with HCC due to hepatitis C. As long as we know, this is the first study that analyzes these signaling pathway in HCV hepatocarcinogenesis. We also evaluated B-Raf protein expression with known anatomopathological features of worse post-transplant or hepatectomy patients outcomes: the size of HCC tumoral lesion, the tumoral differentiation grade and the presence of tumoral vascular invasion^(13,14).

METHODS

Approval

The study was submitted and approved by the Research Ethics Committee of the Clinical Hospital of Federal University of Minas Gerais (COEP ETIC 278/08).

Declared conflict of interest of all authors: none

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Study population

Liver explants from 35 patients that underwent liver transplantation or hepatectomy in the Clinics Hospital (HC-UFGM-EB-SERH) from January/2002 to December/2010 for HCC related cirrhosis were reviewed. Inclusion criteria were as follows: 1) diagnosis of HCV infection by PCR in sera, 2) liver specimens from liver explant available for review and 3) confirmation of histological features of HCC and cirrhosis. Exclusion criteria included any other form of associated liver disease. Twenty-five cases of normal livers obtained from the institution necropsy archives were selected and used as control group. Appropriate institutional review board approved the study. All patients were submitted to a protocol that includes diagnosis of etiology of liver disease and macroscopic evaluation of the explant to evaluated size of tumor nodules.

Preparation of tissues and histological analysis

Sections of 4 µm thickness were performed in paraffin blocks. These sections were fixed later on slides, deparaffinized and stained with Hematoxylin & Eosin technique to choose the most representative specimens of HCC and cirrhosis. Samples of the cirrhotic liver and of the tumor came from the same patients.

Immunohistochemistry

Additional paraffin sections were made and submitted to immunohistochemical technique to investigate protein expression of B-Raf in HCC, cirrhotic and normal hepatocytes. For the application of the technique, the sections were dewaxed in xylene and hydrated with graded ethanol. They were then immersed in a solution of 1 mM EDTA (pH 8.0) and heated to 96°C in vaporizer steamer to antigen retrieval. After cooling and washing the samples with buffer TRIS, 0.05 M Tris-HCL (pH 7.6), endogenous peroxidase activity was blocked with 3% H₂O₂ in water for 10 minutes. Another washing with TRIS was made. Then, the sections were incubated with primary antibodies: anti-B-Raf (Santa Cruz Biotechnology Inc., USA) at a 1:100 dilution. This was followed by incubation with the labeled streptavidin-biotin (LSAB) Kit (DakoCytomation California, Carpinteria, CA). Peroxidase activity was developed with 3,3 diaminobenzidine (DAB) (Sigma, St. Louis, MO) with timed monitoring using a positive control sample. The sections were then counterstained with hematoxylin, dehydrated, and mounted.

Histological analysis

Histological evaluation of all samples was performed by a single expert liver pathologist. HCC was characterized according the following histological features: Predominant Edmonson and Steiner's⁽¹⁵⁾ grade was classified and grouped, I and II as low grade, and III and IV as high grade and the presence of microvascular invasion, defined as the presence of tumor cells in the portal vein, the large capsular vessels or in a vascular space limited by endothelium. Tumor size was obtained by macroscopic evaluation of the liver explant. Immunostaining was evaluated on a scale of 0–3 for intensity as: 0: negative; 1: weak; 2: moderate and 3: strong and 0–4 for the extent of positive staining among hepatocytes as: 0:<5%; 1:5–25%; 2:26–50%; 3:51–75% and 4:>75%. Final score was obtained by multiplying the two individual scores, yielding a range from 0 to 12. Scores of 9–12 were considered strong staining, 6–8 as weak staining and 0–4 as markedly reduced or negative expression. For the purpose of statistical analyses the groups were classified in two and considered strong if scored 9–12 and not strong ≤8.

Statistical analyses

The statistical analysis was performed using the SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). The data correlation was performed using the chi-square association test. Values of $P<0.05$ were considered statistically significant. For data with significance, the odds ratio was calculated in order to quantify this association.

RESULTS

This study included 35 liver explants from liver transplantation or hepatectomy for hepatocellular carcinoma treatment. All cases of HCC in the present study were obtained from cirrhotic liver with HCV as underlying cause. The majority of patients were male (80.6%) and the mean age was 56.4 years (32–79 years). As a control group, we included 25 samples of normal liver. The same way, majority of patients were male (52%) and the mean age was 42.5 years (11–73 years). Demographic and morphological data are summarized in TABLE 1.

TABLE 1. Demographic and morphological data from hepatocellular carcinoma/hepatitis C cirrhosis and normal samples.

	HCC/HCV cirrhosis n (%)	Normal samples n (%)
Gender		
Male	28 (80.0)	13 (52.0)
Female	7 (20.0)	12 (48.0)
Morphological characterization		
Tumor size*		
<20 mm	8 (24.24)	
≥20 mm	25 (74.76)	
Tumor differentiation		
Low grade	24 (68.8)	
High grade	11 (31.4)	
Vascular invasion		
Present	15 (42.9)	
Absent	20 (57.1)	

*n=33 cases. HCC: hepatocellular carcinoma; HCV: hepatitis C.

B-Raf expression in normal, HCV cirrhotic liver and HCC parenchyma

All normal livers showed weak or negative expression for B-Raf. B-Raf was strongly expressed in the HCV cirrhotic parenchyma cytoplasm of 17.1% cases and in 62.9% of HCC samples. Strong B-Raf protein staining was also associated with tumor parenchyma ($P<0.0001$; OR=8.18 (2.62–26.63)). When comparing the strongly positive scores proportion B-Raf proteins in normal livers, in the areas of HCV cirrhosis and in the hepatocarcinoma, there was a statistically significant difference between the groups ($P<0.0001$), as shown in TABLE 2. FIGURE 1 shows an example of cytoplasmic labeling by immunohistochemistry for B-Raf protein in normal liver, HCV cirrhosis and in the HCC.

DISCUSSION

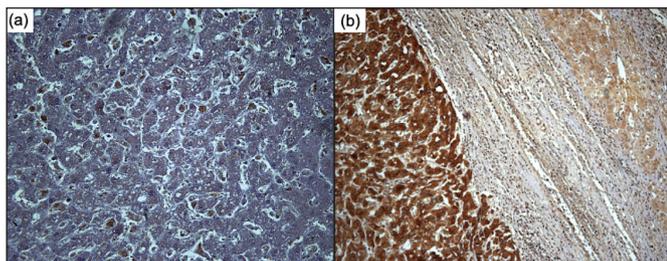


FIGURE 1. B-Raf negative/weak expression in normal liver (a), in HCV cirrhosis (b, up-right) and strong expression in the HCC (b, left). Magnification of 100x.

TABLE 2. Immunostaining score of B-Raf expression in normal liver and hepatocellular carcinoma/hepatitis C cirrhotic parenchyma.

	Normal	Cirrhotic	HCC	P
	n (%)	n (%)	n (%)	OR (CI 95%)
B-Raf				$P^* < 0.0001$
Negative/weak staining	25 (100)	29 (82.9)	13 (37.1)	$P^{***} < 0.0001$
Strong staining	0 (0)	6 (17.1)	22 (62.9)	OR** = 8.18 (2.62–26.63)

*Chi-square test performed for normal vs cirrhotic vs hepatocellular carcinoma. **Chi-square test performed for cirrhotic vs hepatocellular carcinoma (HCC).

Association among B-Raf expression and anatomopathological data

We evaluate the correlation between B-Raf protein expression and morphological markers for HCC. B-Raf scores was not associated with the following predictors of patients' outcome after liver transplantation or hepatectomy: tumor size ($P=0.4427$), tumor differentiation ($P=0.9485$) and vascular invasion ($P=0.2666$), as shown in TABLE 3.

TABLE 3. Correlation between B-Raf protein score in the tumor parenchyma and morphological markers for hepatocellular carcinoma from to hepatitis C liver explant.

Morphological variable	Immunostaining score		P
	Strong staining n (%)	Negative or weak staining n (%)	
B-Raf			
Tumor size*			0.4427
<20 mm	6 (18.18)	2 (6.06)	
>20 mm	15 (45.45)	10 (30.30)	
Tumor differentiation			0.9485
Low grade	15 (42.86)	9 (25.71)	
High grade	7 (20)	4 (11.43)	
Vascular invasion			0.2666
Present	11 (31.43)	4 (11.43)	
Absent	11 (31.43)	9 (25.71)	

*n=33 cases.

Hepatocarcinogenesis is a multistep process initiated by external stimuli that lead to genetic changes in hepatocytes or stem cells, resulting in proliferation, apoptosis, dysplasia and neoplasia. While etiologic factors, including environmental ones, may interfere in carcinogenesis, the mechanism by which each of them induces malignant transformation seems to differ. In the present study, we evaluated the expression of B-Raf protein in surgical specimens with HCC in patients with HCV undergoing liver transplantation or hepatectomy. We found statistically significant difference in immunostaining for B-Raf protein in normal livers, in the areas of HCV cirrhosis and in the hepatocarcinoma in a Brazilian population.

We have shown a progressive enhance of B-Raf expression from normal liver to HCV cirrhotic liver and to HCC samples. Considering that HCV cirrhosis is a known risk factor for HCC, this progressive expression of B-Raf suggests that this protein may be an important factor in the process of hepatocarcinogenesis. Some previous studies have examined the frequency of BRAF gene mutations in HCC samples, with controversial results. Our results are in agreement with Colombino et al. who, in a Italian cohort, demonstrated somatic BRAF mutations in 23% of the HCC samples and a positive correlation of those mutations with the presence of multiple HCC nodules and higher proliferation rates⁽¹⁶⁾. These same authors did not observe this change in the normal areas adjacent to the tumor. Newell et al. also found an overexpression of BRAF gene in hepatocarcinoma⁽¹⁷⁾. On the other hand, Tannapfel et al. and Zuo et al. did not observe a BRAF mutation in their studies that analyzed patients with HCC^(18,19). These results indicates that populations with genetic differences may also present differences in the mechanisms and pathways of hepatocarcinogenesis.

Although the mutation in the BRAF gene is not found in some studies, the change with consequent abnormal activation of the RAF/MAPK/ERK pathway, in which the B-Raf protein participates, is reported as a common phenomenon in hepatocellular carcinoma, being associated with the stage of the tumor⁽¹⁹⁾. According to our findings, changes in the BRAF gene may play an important role in hepatocarcinogenesis in patients with hepatitis C and cirrhosis in this population.

We did not find association between B-Raf protein expression and histopathological markers of tumor progression such as tumor size, tumor differentiation and vascular invasion. Such a find suggest that BRAF gene is more important in tumor initiation than in the differentiation process. Colombino et al. demonstrated an association between gene mutation and the presence of multiple tumor nodules⁽¹⁶⁾. However, the same authors did not find any statistically significant association when comparing the mutation of the gene with the degree of differentiation or size of the tumor, which is in accordance with our findings.

In our study we used immunohistochemistry for B-Raf protein, a fast, inexpensive technique that can be used in the routine of most surgical pathology services. This choice was made because immunohistochemistry could be used as a marker of prognosis if any association between B-Raf protein expression and histopathological markers of tumor malignance was found. We did not find any association. The fact that the gene sequencing was not carried out, as well as the sample consisting only of patients with HCC due to cirrhosis associated with virus C are the main limiting factors of these result. However, we have shown a pro-

gressive enhance of B-Raf expression from normal liver to HCV cirrhotic liver and to HCC samples. Future studies are needed to validate the role BRAF gene in liver carcinogenesis in a larger group of patients with different cirrhosis etiologies and environmental exposures. Analyzing genetic and epigenetic alteration as well as different molecular pathways involved in the development of HCC is a critical process toward identifying potential new therapies⁽²⁰⁾ and also making a genome-based classification of risk factors and prognosis.

CONCLUSION

We found B-Raf protein immunostaining difference in normal livers, in the areas of HCV cirrhosis and in the hepatocarcinoma. We did not find association between B-Raf protein expression and histopathological markers of tumor progression. Our data suggests that BRAF gene pathway may play an important role in initial HCC carcinogenesis. Larger studies are needed to validate these observations.

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Authors' contribution

Methodology: Garcia PP, Vidigal PVT. Formal analysis: Garcia PP, Albuquerque RM, Vidigal PVT. Funding acquisition: Vidigal PVT. Project administration: Vidigal PVT. Writing original draft: Garcia PP, Albuquerque RM. Writing review, conceptualization, editing and approval of final manuscript: all authors.

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RESUMO – Contexto – A hepatocarcinogênese é um processo de múltiplas etapas que leva a alterações genéticas nos hepatócitos, resultando em neoplasia. No entanto, os mecanismos da transformação maligna parecem diferir amplamente. Conhecer os mecanismos da carcinogênese é fundamental para o desenvolvimento de novos métodos de tratamento e prevenção. **Objetivo** – O objetivo deste estudo é analisar a imunorexpressão da proteína B-Raf em explantes de carcinoma hepatocelular (CHC), em tecido cirrótico relacionado à hepatite C adjacente e em fígados normais. Também analisamos a imunorexpressão com características histopatológicas prognósticas relacionadas ao CHC. **Métodos** – Foram estudados fígados de 35 pacientes com CHC relacionado à cirrose por vírus C submetidos a transplante hepático ou hepatectomia no Hospital das Clínicas – UFMG e 25 fígados normais de arquivos de necropsia. Os tumores foram classificados de acordo com tamanho do tumor, invasão vascular e grau de diferenciação. A expressão de B-Raf foi determinada por imunohistoquímica. **Resultados** – B-Raf foi fortemente expresso no citoplasma do parênquima cirrótico em 17,1% dos casos e em 62,9% das amostras de CHC. A forte expressão da proteína B-Raf foi associada ao tecido tumoral ($P < 0,0001$; $OR = 8,18$ (2,62–26,63)). Todos os fígados normais apresentaram expressão fraca ou negativa para B-Raf. Não houve associação significativa entre os escores B-Raf e o grau de diferenciação do tumor ($P = 0,9485$), tamanho do tumor ($P = 0,4427$) ou invasão vascular ($P = 0,26666$). **Conclusão** – Encontramos diferença na imunorexpressão da proteína B-Raf em fígados normais, nas áreas de cirrose por HCV e no hepatocarcinoma. Não encontramos associação entre a expressão de B-Raf e marcadores histopatológicos de progressão tumoral. Nossos dados sugerem que o B-Raf pode desempenhar um papel importante na carcinogênese inicial do CHC. Estudos maiores são necessários para validar essas observações.

Palavras-chave – Carcinoma hepatocelular; B-Raf; vírus da hepatite C.

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