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ASSOCIATION BETWEEN MURINE DOUBLE MINUTE 2 – T309G polymorphism and recurrence of hepatocellular carcinoma after surgical treatment

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ABSTRACT - ***Background*** **-** Discovery and incorporation of biomarker panels to cancer studies enabled the understanding of genetic variation and its interference in carcinogenesis at molecular level. The potential association between single nucleotide polymorphism (SNP) 309 and increased development of tumors, such as hepatocellular carcinoma, has been subject to several studies. This is the first study on this association conducted in Brazil. ***Methods*** **-** 62 cases of cirrhotic patients with hepatocellular carcinoma surgically treated by partial hepatectomy (HPT) or by liver transplantation (LTX) from 2000 to 2009 at Santa Casa Hospital Complex, in the city of Porto Alegre, were retrospectively analyzed. Tumor samples from surgical specimen were collected and prepared for study in paraffin blocks. ***Results*** **-** Overall survival was 26.7 months in the HPT group and 62.4 months in the LTX group (P<0.01). Overall tumor recurrence was 66.7% in the HPT group (10/15) and 17% in the LTX group (8/47) (X^2=13.602, P<0.01). Alpha-fetoprotein levels >200ng/mL, microvascular invasion and histological grade were associated with tumor recurrence (P<0.01). Recurrence rates in each surgical group and analysis of factors associated with tumor recurrence, when stratified for each genotypic pattern, were both not statistically significant. ***Conclusion*** **-** G/G genotype was not associated with tumor recurrence after surgical treatment and it did not show any correlation with other prognostic factors.

HEADINGS - Proto-oncogene proteins C-MDM2. Single-stranded conformational polymorphism. Hepatocellular carcinoma. Biological tumor markers

INTRODUCTION

With advancements in molecular epidemiology, the concept of individual susceptibility factors modulating environmental factors became the focus of various research efforts[20]. Discovery and incorporation of biomarker panels to cancer studies enabled the understanding of genetic variations and their interference in carcinogenesis[23].

DNA (deoxyribonucleic acid) repair is a crucial mechanism in guaranteeing genome integrity[28] and P53 protein plays an essential role in this process. Identified for the first time in 1979, P53 was later described as "guardian of the genome" for blocking inappropriate cellular proliferation and directing cells to apoptosis[3]. Other proteins were later recognized as p53 regulators, such as Murine Double Minute 2 (MDM2). This protein acts as a negative regulator by reducing P53 cellular activity and promoting its degradation[5, 15].

MDM2 T309G single nucleotide polymorphism (SNP) is the substitution of a thymine for a guanine in the 309 position of the MDM2 gene. Since the first publication regarding this polymorphism in 2004[4], researchers have been trying to document the association between MDM2 T309G SNP and increased tumor formation. MDM2 hyperfunction in consequence of SNP309 would lead to a reduction in p53 levels and activity. MDM2-p53 interaction has already been targeted by several studies about cancer behavior[2, 11, 19].

Hepatocellular carcinoma (HCC) is a high incidence malignancy and it is responsible for high mortality rates all over the world[12]. The MDM2-p53

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association is still uncertain considering this type of tumor. A number of risk factors and prognostic factors are known for this type of carcinoma, and surgical resection remains as a main treatment method[10, 14, 18]. However, correlations between genetic alterations, tumor recurrence and prognostic factors are not well established.

This is the first study conducted in Brazil to analyze the influence of Murine Double Minute 2 - T309G polymorphism on tumor recurrence in cirrhotic patients with HCC after standard surgical treatment. Associations between numerous genotypes and known prognostic factors, such as number of tumors, preoperative alpha-fetoprotein levels, tumor size, histological grade and microvascular invasion are also to be reported. The Institutional Ethics Committee approved the study under the report 350.488 in 07/17/2013.

METHODS

An observational and retrospective study was conducted. Cirrhotic patients who underwent surgery for hepatocellular carcinoma from 2000 to 2009 at Santa Casa Hospital Complex in Porto Alegre, South Brazil were selected. Tumor samples from surgical specimen (explanted liver or hepatic segment) were collected and prepared for study in paraffin blocks. All patients had histological confirmation of cirrhosis and hepatocellular carcinoma through anatomopathological study.

From 124 initial samples, 8 samples were discarded owing to insufficient material and 24 samples were rejected for excess of necrosis. From the remainder 92 samples, 11 samples were eliminated for failure in DNA extraction and 14 samples were excluded for failure in DNA amplification. From the final 67 samples, 62 subjects were selected, after the removal of 3 patients due to incomplete medical records and 2 patients due to death in relation to surgery.

The paraffin blocks were sliced in 4μm fragments. After preparation, we extracted DNA using Bioneer extraction kit (Bionner Corporation, EE.UU.), accordingly to manufacturer instructions. We evaluated DNA quantity and integrity by sample, using NanoDrop (NanoDrop Technologies, Wilmington, EUA). DNA from each sample was extracted, quantified and stored at -20ºC.

Molecular identification of MDM2 T309G polymorphism was executed through polymerase chain reaction (PCR) technique. TaqMan essay and Step One Plus thermal cycler (Applied Biosystems, Foster City, CA) were used for this step. A pair of primers was employed to identify the DNA sequence that contained the polymorphism (Foward 5'CGGGAGTTCAGGGTAAAGGT 3' e Reverse:5' ACAGGCACCTGCGATCATC 3'). Two probes were employed, the first to identify the allele that enclosed the polymorphism and the second to identify the allele without the polymorphism (VIC-5'-CTCCCGCGCCGAAG-3' and FAM-5'-TCCCGCGCCGCAG-3') (primers and probes from Applied Biosystems, Foster City, CA).

Patient-related data were collected in institutional medical records. The last measure of alpha-fetoprotein (AFP) levels before surgery was the one considered and it was stratified in categories due to the variability of values. Viral hepatitis infection was confirmed by specific serology. Number of tumors, size of the largest tumor, vascular invasion and histological grade were documented accordingly to anatomopathological study. Histological grade was classified as well-differentiated (1), moderately differentiated (2) and poorly differentiated (3). Tumor recurrence evaluation was defined based on standardized radiologic criteria (triphasic computed tomography or magnetic resonance imaging) or liver biopsy.

Statistical analysis was executed using PSPP 0.8.4 software. Chisquare test was conducted considering a significance level of 5%.

RESULTS

Table 1 presents the sample. Of 62 patients, 52 patients were male (83.9%) and 10 patients were female (16.1%).

TABLE 1. Characteristics of the sample and results of anatomopathological study

Variables	n=62
Age (years)	65 (50-84)
Sex, nº (%)	
Male	52 (83.9)
Female	10 (16.1)
AFP, ng/mL (%)	
≤50.0 ng/mL	42 (67.7)
50.1 a 200.0 ng/mL	5 (8.1)
>200.0 ng/mL	15 (24.2)
Viral Hepatitis, nº (%)	
VHC	54 (87.1)
VHB	2 (3.2)
VHC + VHB	1 (1.6)
Number of nodules (%)	
1	40 (64.5)
2	14 (22.6)
3	5 (8.1)
≥4	3 (4.8)
Size of largest nodule	
Up to 3 cm	40 (64.5)
3.1 a 5 cm	18 (29.0)
> 5 cm	4 (6.5)
Surgery, nº (%)	
Partial hepatectomy	15 (24.2)
Liver transplantation	47 (75.8)
Histological grade, nº (%)	
Well-differentiated (1)	10 (16.1)
Moderately differentiated (2)	36 (58.1)
Poorly differentiated (3)	16 (25.8)
Microvascular invasion, nº (%)	
Yes	22 (35.5)
No	40 (64.5)
Genotype, nº (%)	
G/G	22 (35.5)
T/G	17 (27.4)
T/T	23 (37.1)

Mean age was 65 years (50-84 years). Hepatitis C virus was present in 87.1% of patients. Stratification by AFP levels showed that 67.7% of patients presented values fewer than or equal to 50ng/mL. Single tumor was observed in 64.5% of patients. Size of the largest tumor was fewer than or equal to 3 cm in 40 patients (65.4%); 22 participants displayed genotype G/G, 17 participants displayed genotype T/G and 23 the T/T one.

Overall survival was 26.7 months in the HPT group and 62.4 months in the LTX group (P<0.01). Global recurrence rate was 29% (18 patients - 10 patients from the hepatectomy group and 8 patients from the transplantation group). There was 66.7% of global recurrence in the HPT group (10/15) and 17% of global recurrence in the LTX group (8/47) (X^2=13.602, P<0.01) (Figure 1).

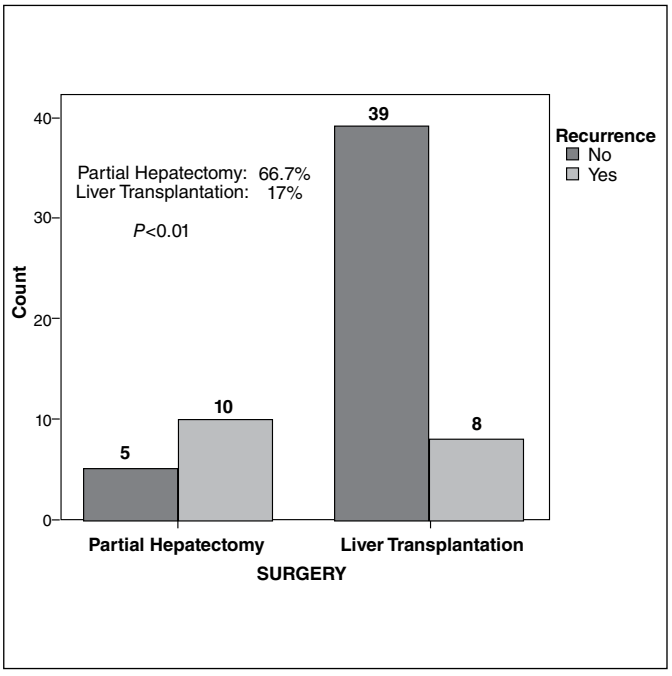

FIGURE 1. Tumor recurrence stratified by surgery

Regarding recurrence rate and preoperative AFP levels between surgical groups, in the LTX group (eight patients), 50% had AFP levels >200ng/mL, 37.5% levels <50ng/mL and 12.5% values between 50.1 and 200ng/mL (P=0.042). In the HPT group (10 patients), 70% of patients had AFP levels >200ng/mL, 20% levels between 50.1 and 200ng/mL and 10 had <50ng/mL (P<0.024).

Considering microvascular invasion, 83.3% of patients with recurrence presented it in anatomopathological study (P<0.01). In the HPT group, 66.7% had microvascular invasion and from these, 80% presented recurrence (P>0.05). In the LTX group, 25.5% of patients had microvascular invasion and from these, 58.3% presented recurrence (P<0.01).

Regarding tumor differentiation, from the 18 patients with recurrence, 10 (55.6%) presented poorly differentiated tumors, 6 (33.3%) presented moderately differentiated tumors and 2 (11.1%) well-differentiated tumors (P=0.034).

In the analysis of groups by genotype, in the HPT group 50% of patients with recurrence presented G/G genotype, 30% presented T/T genotpe and 20% the T/G genotype (P=0.221). In the LTX group, 50% of patients with recurrence carried G/G genotype, 37.5% the T/G genotype and 12.5% the T/T one (P>0.05).

Study of prognostic factors related to hepatocellular carcinoma revealed that 45.5% of patients were G/G genotype, 31.8% were T/G genotype and 22.7% the T/T genotype among the 22 patients with microvascular invasion. In the group with AFP levels >200ng/mL, 40% of patients presented T/G genotype, 33.3% presented G/G genotype and 26.7% the T/T genotype (P>0.05). Considering the 16 patients with poorly differentiated tumors, 43.8 were G/G genotype, 37.4% were T/T and 18.8% of patients were T/G (P>0.05). For individuals with 1 tumor, distribution was 42.5% of patients with T/T genotype, 27.5% with T/G genotype and 30% with the G/G one. Finally, among patients with tumors up to 3 cm, proportion to each genotype was 37.5% with G/G, 35% with T/G and 25.5% of patients with T/T genotype (P>0.05) (Table 2).

TABLE 2. Distribution of prognostic factors for each genotype

Prognostic factors	Genotype TT	TG	GG	*P* value
AFP, ng/mL				
≤50.0	18	9	15	
50.1 a 200.0	1	2	2	
>200.0	4	6	5	>0.05
Microvascular invasion				
Yes	5	7	10	>0.05
No	18	10	12	
Histological grade				
Well	4	2	4	
Moderately	13	12	11	
Poorly	3	6	7	>0.05
Number of nodules				
1	17	11	12	
2	2	5	7	
3	3	0	2	
≥4	1	1	1	>0.05
Size largest nodule				
Up to 3.0 cm	11	14	15	
3.1 a 5.0 cm	10	3	5	
> 5.0 cm	2	0	2	>0.05

DISCUSSION

The identification of risk factors for prognosis in HCC after surgical treatment has been a constant aim of research. The recognition of genetic alterations and specific genotypes that may directly interfere in outcome and in factors known to be related to unfavorable results are of significant relevance.

Regarding type of surgery, hepatectomy was more closely related to tumor recurrence when compared with liver transplantation. Even though surgical resection offers a chance of cure, recurrence rates are high after surgery and cumulative recurrence incidence in 5 years varies from 40 to 70% in several studies[6]. In a study conducted in Santa Casa Hospital Complex in Porto Alegre in 2008, Silva et al.[22] analyzed cirrhotic patients with hepatocellular carcinoma after surgical resection and observed a mean survival of 33.5 months; 61.9% of survival in 1 year, 16.67% in 3 years and 11.11% in 5 years. A tumor recurrence rate of 68.4% was obtained, mostly in the first year after surgery. A mean survival of 26.7 months and a recurrence rate of 66.7% were found in our study.

Other factors related to tumor recurrence in our series were preoperative AFP levels >200ng/mL, poorly differentiated histological grade and microvascular invasion. These results match the current literature, which states that tumors size larger than 5cm, less differentiation, elevated AFP levels and vascular invasion are related to poor prognosis[6]. Park et al.[17] reported similar parameters in a study conducted in Korea, whereas AFP levels >400ng/mL, HCC>5cm, multiple tumors and vascular invasion were related to less favorable outcomes. On the other hand, Shindoh et al.[21] observed that long-term survival was not affected by microvascular invasion among patients presenting small HCC tumors (fewer than 2 cm); the opposite happened in cases with tumors larger than 2 cm.

Divergence in our study was observed considering number of tumors and size of the largest tumor: both were not associated with recurrence – in opposite to what was revealed by the studies listed in the last paragraph. A cause for this abnormality could be the small sample in our study. In literature, the number of tumors is conspicuously a poor prognosis factor after surgical treatment and it is associated to high recurrence rates and a low life expectancy[25]. Also, in our series most patients had a single tumor, smaller than 3 cm, making the comparison between groups more difficult to proceed with. In 2004, Chen et al.[6] demonstrated an association between multiple initial lesions, short recurrence interval and adverse outcomes.

Regarding the T309G polymorphism, current literature shows conflicting results. In the last few years, various researchers have been trying to evaluate association between SNP T309G and hepatocellular carcinoma. In 2007, Leu et al.[16] analyzed the MDM2-SNP-309 genotype of 58 patients with HCC and hepatitis B/C in Taiwan, in comparison with healthy controls. A similar proportion of the homozygous genotype MDM2-SNP 309 (G/G) was found in cases and controls. No difference in prevalence of G/G and T/T genotype was found among patients presenting HCC. On the other hand, between 2001 and 2003, Dharel et al.[7] studied 435 patients with hepatitis C – 187 patients with HCC and 248 patients without HCC – and obtained DNA samples from 48 healthy controls to estimate genetic distribution of SNP309 in Japan. G/G genotype proportion in patients with HCC was significantly higher than in healthy individuals. G/G genotype was independently associated to HCC when compared with T/T genotype.

In 2011, Di Vuolo et al.[8] analyzed the genotypic distribution of 61 patients with HCC and viral hepatitis in comparison with 122 healthy controls. Frequency of MDM2-SNP309 G/G and T/G genotypes was significantly higher in patients presenting HCC. Furthermore, when the MDM2-SNP309 T/T group was used as a reference, G/G and T/G genotypes were associated with a higher risk of HCC.

The potential role of the MDM2-SNP polymorphism in HCC development was analyzed in other studies. MDM2-SNP allele was associated with a higher risk of developing HCC in Korean patients with chronic hepatitis B[27], Moroccan patients with viral hepatitis[9] and Turkish patients[1]. In 2011, Jin et al.[13] published a meta-analysis of these studies, including 738 cases and 1062 controls, in order to evaluate the association of MDM2-SNP-309 G allele with HCC. The grouped odds ratio was 1.57 (CI 95%; 1.36-1.80) for G allele when compared with T, indicating that this SNP may be used as a predictive molecular marker for HCC screening in high-risk populations.

Tang et al.[24], in a recent meta-analysis including 10 studies – 2.243 cases and 3.471 controls – also detected an association between T309G polymorphism and a higher risk of hepatocellular carcinoma in all three genetic models (G vs. T: OR=1.39, 95% CI 1.17-1.64; GG vs TT: OR=1.87, 95% CI 1.34–2.62, $P<0.001$; GG/GT vs TT: OR=1.61, 95% CI 1.24–2.08, $P<0.001$).

Concerning the association between SNP309 and prognostic factors related to HCC after surgical treatment, current literature is scarce. A single study conducted by Yang et al. in 2003[26] contemplated this association and noticed patients presenting G/G genotype had disease-free survival and global survival similar to other genotypes.

In our study, 50% of patients with recurrence in each group were G/G genotype carriers. This difference, even though epidemiologically expressive, was not statistically significant, possibly because of the small sample. Regarding prognostic factors, most patients with microvascular invasion and poorly differentiated tumors presented G/G genotype (45.5% and 43.8%, respectively), a result that express a tendency, despite not statistically significant. Analysis of microvascular invasion revealed T/T genotype was present in most patients (45%) who did not present this feature – reinforcing once more the possible influence of the mutation to the occurrence of invasion. Genotypic distribution of the other features evaluated varied and did not follow a pattern according to genotype.

This is the first study concerning T309G in Brazil and evaluating the polymorphism association with hepatocel-

lular carcinoma and its recurrence after surgical treatment. As retrospective study, it is vulnerable to bias inherent to this design. As the variables studied were laboratorial measures and pathology results, data collection was facilitated. Working with patients from a single center was another limiting factor, as was the reduced sample.

Authors' contributions

Data collection: Teixeira UF, Marchry MC, Izaguirre AGC. Search literature: Teixeira UF, Izaguirre AGC. Data analysis: Marchry MC, Cerski CT, Brandão ABM, Fontes PRO. Manuscript writing: Teixeira UF, Brandão ABM, Fontes PRO.

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RESUMO - ***Contexto*** **-** A descoberta e incorporação de painéis de biomarcadores aos estudos do câncer permitiram o conhecimento de variações genéticas e sua interferência no processo de carcinogênese. A possibilidade de associação do polimorfismo de nucleotídeo simples T309G do gene MDM2 com o aumento da formação de tumores, dentre eles o hepatocarcinoma, tem sido alvo de diversos estudos. ***Objetivo*** **-** Analisar a influência do polimorfismo T309G do gene MDM2 na recidiva tumoral de pacientes cirróticos com hepatocarcinoma submetidos a tratamento cirúrgico. ***Métodos*** **-** Foram analisados retrospectivamente pacientes cirróticos com carcinoma hepatocelular submetidos a tratamento cirúrgico (hepatectomia parcial ou transplante hepático) no período de 2000 a 2009, na Santa Casa Hospital Complex in Porto Alegre, South Brazil. Foram coletadas amostras de fragmentos tumorais da peça operatória (fígado explantado ou segmento hepático), as quais foram preparadas para estudo em bloco parafinado. ***Resultados*** **-** A sobrevida global foi de 26,7 meses para o grupo hepatectomias e 62,4 meses para o grupo transplante hepático (P<0,01), havendo 66,7% de recidiva global no grupo hepatectomias (10/15), e 17% no grupo transplante hepático (8/47) (X^2=13,602, P<0.01). Níveis de AFP>200ng/mL correlacionaram-se com a recidiva tumoral em ambos os subgrupos cirúrgicos. Observou-se que 83,3% dos pacientes com recidiva também apresentaram invasão microvascular ao exame anátomo-patológico (P<0,01). Não houve significância estatística quando a recidiva neoplásica foi avaliada para os diferentes genótipos e analisada para cada subgrupo cirúrgico. A análise dos fatores prognósticos relacionados à recidiva do hepatocarcinoma, quando estratificada para cada padrão genotípico, também não se mostrou significante. ***Conclusão*** **-** O nosso estudo revelou que o genótipo G/G não esteve associado à recidiva tumoral após o tratamento cirúrgico, seja nas hepatectomias parciais ou transplante hepático. Além disso, a presença desse genótipo não mostrou correlação com os fatores prognósticos estudados.

DESCRITORES - Proteínas proto-oncogênicas C-MDM2. Polimorfismo conformacional de fita simples. Carcinoma hepatocelular. Marcadores biológicos de tumor.

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