RESEARCH ARTICLE

Crosstalk between EGFR and p53 in Hepatocellular Carcinoma

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Abstract

Background: Hepatocellular carcinoma (HCC) is one of the most frequent cancers worldwide, with a high mortality. Most patients present with late stage disease, when the treatment options are limited to systemic chemotherapy. The purpose of our study was to evaluate the significance of p53 and EGFR expression in HCC, and to determine whether these two markers correlate with conventional parameters of prognosis. Materials and Methods: Our study included a total of 45 patients, diagnosed histopathologically with HCC. Clinicopathological data including sex, age, tumor necrosis, tumor size, histologic grading, tumor stage, the presence of cirrhosis and chronic hepatitis, were recorded from the Institute database. Three independent microscopic fields were selected for each sample and all the tumor cells within each microscopic field were counted, and then the positive percent of p53 cells were calculated. Three staining patterns were recognized: diffuse, heterogenous and focal. The intensity of EGFR staining was scored on a scale of 0-3+: 0 no staining; 1+ when a weak membrane staining was observed; 2+ when membrane staining is more intense than in 1+, but less than 3+, and 3+ when intense dark brown staining delineated the membrane. To determine the relationship between EGFR expression and p53, we performed double staining in the same HCC specimens. Results: By immunohistochemical staining, p53 protein was detected in tumor cell nuclei in 20 HCCs (44%). We found a significant correlation between the intensity of p53 expression and the histological grade (p=0.008). EGFR expression was detected in 17 (38%) cases, linked to histological grade (p=0.039). Moreover, the intensity of p53 expression was significantly correlated with EGFR intensity (p=0.014). Conclusions: Our results suggest that overexpression of p53 and EGFR plays an important role in hepatocarcinogenesis and contributes to more advanced disease. These markers are not only valuable predictors of prognosis in HCC, but they are also rational targets for new anti-tumor strategies.

Keywords: Epidermal growth factor receptor - p53 - hepatocellular carcinoma - immunohistochemistry

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Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent cancers worldwide, with high risk in Asia and Africa and less common, but with increasing incidence, in Western-developed countries. The worldwide incidence of HCC is estimated at 600.000 cases annually. Despite the recent advances in molecular mechanism of hepatocarcinogenesis, the prognosis of HCC still remains poor (Wei et al., 2013). The incidence and mortality of HCC are estimated to be double in the next two decades (Rampone et al., 2009).

In the majority of cases, HCC is associated with a chronic liver disease as cirrhosis or viral hepatitis, which is regarded as pre-neoplastic conditions for liver cancer (Xu et al., 2012). Other factors that increase the risk for HCC are aflatoxin exposure, cigarette smoking, chronic alcohol abuse, α1-antitrypsin deficiency and hemochromatosis (Andreas et al., 2011). Some studies demonstrated that obesity and diabetes mellitus might be involved in HCC development (Gao et al., 2012).

The prognostic and predictive factors already known in HCC are tumor stage and grade, angiolymphatic invasion and high AFP levels (Hassan et al., 2002; Vlasoff et al., 2002). For patients with early HCC that may benefit from liver resection, the survival rate at 5 years increased to 51.8% (Zhou et al., 2013). Unfortunately, most of the patients with HCC present in a late stage disease, when the treatment options are limited to chemoembolization or to systemic chemotherapy (Ito et al., 2001). The efficacy of the conventional cytotoxic drugs is poor and new therapeutic targets are imperative.

The tumor-suppressor gene p53 holds an essential role in preserving stability by preventing genome mutation. The major function of the gene is blockage of cell cycle progression in response to DNA damage (Xu et al., 2012).

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Mutation of the p53 is the most commonly detected anomaly in human cancers and immunohistochemical overexpression of p53 protein was correlated with a poor prognosis in various human malignancies including HCC (Hsia et al., 2000; Qin et al., 2001; Azlin et al., 2014).

Epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptor tyrosine kinases that plays an important role in tumor progression. In addition to EGFR (Her 1), the other receptors from Erb B family include Her2/c-neu, Her3 and Her4. In HCC, EGFR contributes to the proliferation, resistance to apoptosis and to an invasive behavior of the tumoral cells (Berasain et al., 2011). Overexpression of EGFR was found in several human cancers, including HCC, indicating that EGFR inhibitors are rational therapeutic agents (Cervello et al., 2012).

In our study, we assessed the expression of EGFR and p53 by immunohistochemistry and we analyzed the relationship between these two markers and their correlation with the clinical and pathologic parameters.

Materials and Methods

Patients and tissue samples

Our study included a total of 45 patients, diagnosed histopathologically with HCC, in the period between 2002-2012, who underwent partial hepatectomy in the Department of Surgery of the Regional Institute of Gastroenterology and Hepatology "Prof. Dr. Octavian Fodor", Cluj-Napoca. The clinicopathological data including sex, age, tumor necrosis, histologic grading, tumor stage, hepatitis status, the presence of cirrhosis and tumor size were recorded from the institute database and from the pathology reports.

Specimens were fixed in 10% buffer formalin and paraffin embedded. In order to assess the tumor grade and stage, histological sections were cut at 5-μm thickness and stained with the conventional hematoxylin and eosin method.

Immunohistochemistry

Five μm thick sections were performed from each case. The dewaxing and rehydration of the sections was followed by heat-induced epitope retrieval in citrate buffer pH6 for 30 minutes (with PT link module, Dako Cytomation, Denmark). The immunohistochemical technique continued with the blocking of the endogeneous peroxidases, using hydrogen peroxide 3%. Incubation with the EGFR primary antibody (clone EGFR 25, RTU, Novocastra, Newcastle upon Tyne, UK) and p 53 primary antibody (clone DO-7, Leica Biosystems, Newcastle uponTyne, UK) had a duration of 30 minutes. NovoLink Max Polymer Detection System, was applied for 30 minutes, as visualisation system. The Bond Polymer Refine Detection System (Leica Biosystems, Newcastle uponTyne, UK) was used for visualisation. 3,3 diamino-benzidine dyhidrochloride was applied as chromogen and hematoxyline was used for counterstain.

Immunohistochemical study included double immunostaining p53/EGFR. Heat-induced epitope retrieval with pH 6.0 solution (Leica Biosystems, Newcastle uponTyne, UK), for 30 minutes was followed by endogenous peroxidase blocking (3% hydrogen peroxide-5 minutes) and incubation with primary antibodies (p53, clone DO-7, Novocastra, Newcastle UponTyne, ready to use, 30 minutes). NovoLink Max Polymer Detection System The Leica Biosystems, Newcastle uponTyne, UK was used as visualization system and 3,3 - diaminobenzidine as chromogen. Immunohistochemical technique continued with endogenous peroxidase blocking with 3% hydrogen peroxide for 5 minutes, incubation with the second antibody (EGFR.25, Novocastra, Newcastle UponTyne, ready to use, 30 minutes), visualization (Dako REAL Detection System, APAAP, Mouse, Dako Glostrup Denmark, 30 minutes) and Warp Red chromogen application for 10 minutes (Biocare Medical, LLC, Concord, CA 94520, USA). Counterstaining was performed with Lille's hematoxylin. The entire immunohistochemical procedure was performed with DakoAutostainer Plus (DakoCytomation).

Image acquisition and analysis were performed using Nikon Eclipse E 600 microscope and Lucia G software for microscopic image analysis.

The local research ethics committee approved the protocol of the study and informed consent was obtained from all subjects according to the World Medical Association Declaration of Helsinki.

Evaluation of p53 staining

Three independent microscopic fields (×400) were selected for each sample and all the tumor cells within each microscopic field were counted, and then the positive percent of p53 cells were calculated. We considered a positive reaction only in the presence of immunostained nuclei in brown shades, in more than 5% of the tumoral nuclei. Three staining patterns were recognized: diffuse nuclear staining which express uniform diffuse positivity on tumoral cells (>50%), heterogeneous nuclear staining which express areas of strong positivity alternating with areas of weak positivity (10%-50%) and focal nuclear staining which express a small nests or isolated tumoral cells (>5%). Also, the intensity of reaction was assessed as low (+), moderate (++) or intense (+++).

Evaluation of EGFR staining

Intensity of staining was scored on a scale of 0-3+: 0- no staining, 1+ when a weak membrane staining was observed; 2+ when membrane staining is more intense than in 1+, but less than 3+, and 3+ when intense brown staining delineated the membrane.

Double immunohistochemistry

Cases that showed both p53 and EGFR positivity, were subject for double immunostaining; p53 was stained in brown while EGFR was stained in red.

Statistical analysis

Statistical analysis was performed to determine the relationship between the clinical parameters of gender, age, presence of cirrhosis, chronic hepatitis, tumor stage, histological grading, tumor size and presence of tumor necrosis and the 2 immunohistochemical markers using

Pearson's chi-square test. The Spearman correlation was employed to examine the relationship between the expression of p53 and EGFR. P-values of less than 0.05 were considered statistically significant. All statistical analysis was performed using the SPSS 22.0 software for Windows 8.

Results

Clinical characteristics

Our study included samples from 45 patients composed of 32 males and 13 females, with ages between 29-77 years.

In relation to tumor size, HCCs were divided into 2 categories: tumors smaller than 5 cm and tumors greater than 5 cm. From all of 45 HCCs, 12 tumors were larger than 5 cm and 33 of tumors were smaller than 5 cm.

According to Edmondson and Steiner system (Edmondson et al., 1954), tumor grade was divided into three groups: well differentiated (grade I-4 cases), moderately differentiated (grade II-20 cases) and poorly differentiated (grades III and IV-21 cases).

Tumor stages were classified according to TMN classification of tumors of the liver as I (12 cases), II (18 cases), III (14 cases), and IV (1 case). The nontumoral liver showed cirrhosis in 17 (38%) patients and chronic hepatitis in 18 (40%).

p53 and EGFR expression in HCC

By the immunohistochemical stain, p53 protein was detected in the tumor cell nucleus in 20 HCCs (44%), including diffuse positive in 7 (35%) cases, heterogeneous in 5 (25%) cases, and focal in 8 (40%) cases. Those without p53 expression or p53 expression positive in less than 5% of tumor cells were considered as negative (25 cases, 56%). From all of 45 HCCs, analysis of p53 positivity revealed intense positivity in 8 (40%) cases, moderate positivity in 7 (35%) cases and week positivity in 5 (25%) cases (Figure 1 A). p53 expression showed positivity in 43.7% of males and 46.1% of females and appeared to be more frequently in young patients (53.8% in patients <60 years and 40.6% in patients >60 years), but without significant correlations.

In reference to the tumor stage, the expression of p53 presented a maximum score in stage II (61%) and stage III (38%). p53 tented to be overexpressed in poorly differentiated HCCs. HCCs with p53 showed positivity in 12 (50%) HCCs larger than 5 cm and in 33 (42%) HCCs smaller than 5 cm.

The expression pattern of EGFR was membranous and cytoplasmic, but we considered a positive reaction only when the membrane staining was observed. EGFR expression was detected in 17 (38%) cases. Of the 17 HCCs showing EGFR overexpression, 3 (18%) showed 1+ staining, 9 (53%) showed 2+ staining, and 5 (29%) showed 3+ staining. EGFR was positive in 37.5 % males and in 38.4 females and as p53, EGFR tented to be expressed in young patients (58.8% in patients <60 years and 31.2% in patients >60 years).

Correlation of p53, EGFR and clinicopathological features

Clinicopathological data including sex, age, presence of cirrhosis, chronic hepatitis, tumor stage, histological

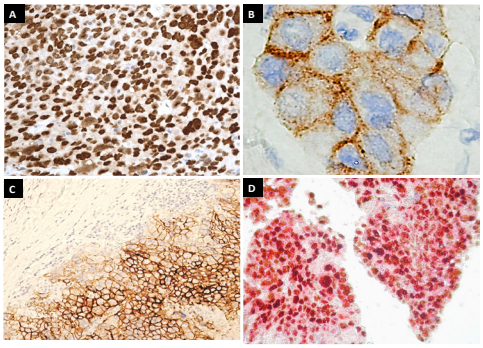

Figure 1. A) Poorly Differentiated HCC Showing Positivity for p53 in the Nuclei of Malignant Cells, 400x; B) EGFR Membranar Staining at High Magnification; C) Strong and Complete EGFR Membranar Staining (score 3+), 200x; D) Double Immunostaining of p53 (brown) and EGFR (red) in HCC, 400x

Table 1. Expression of p53 and EGFR in relation with clinicopathological parameters

Clinicopathological parameters		No. of cases	P53 positive cases (%)	EGFR positive cases	P53 p-value	EGFR p-value
Gender	Males	32	14 (43.7%)	12 (37.5%)	>0.5	>0.5
	Females	13	6 (46.1%)	5 (38.4%)	>0.5	>0.5
Age	<60 years	13	7 (58.8%)	7 (58.8%)	>0.5	>0.5
	>60 years	32	13 (40.6%)	10 (31.25%)	>0.5	>0.5
Grade	Well differentiated (gr.I)	4	0	0	0.008	0.025
	Moderately differentiated (gr.II)	20	7 (35%)	6 (30%)		
	Poorly differentiated (gr.III, IV)	21	13 (62%)	11 (52.3%)		
Stage	I	12	4 (33%)	3 (25%)	>0.5	>0.5
	II	18	11 (61%)	8 (44.4%)		
	III (A, B, C)	14	5 (36%)	6 (42.8%)		
	IV	1	0 (0%)	0 (0%)		
Tumor size	<5cm	33	14 (42%)	13 (39.3%)	>0.5	
	>5 cm	12	6 (50%)	4 (33.3%)		
Tumoral necrosis		17	7 (41%)	6 (35.2%)	>0.5	
Cirrhosis		17	8 (47%)	9 (52.9%)	>0.5	
Chronic hepatitis		18	8 (44%)	7 (38.8%)	>0.5	

grading, tumor size and presence of tumoral necrosis were analyzed and correlated with p53 and EGFR expression (Table 1).

We have found a significant correlation between the intensity of p53 expression and the histological grade (p=0.008). p53 stain did not show a relation with the other clinicopathological parameters.

EGFR expression was significant correlated with the histological grade (p=0.025), but not with the other clinicopathological parameters examined. The more advanced histologic grades were correlated with higher EGFR intensity (Figure 1 B and C). Double immunostained revealed that p53 and EGFR positivity often occurs in the same tumor cells (Figure 1D).

An interesting correlation was noted between p53 expression and EGFR intensity (p=0.014).

Discussion

Hepatocarcinogenesis is a multi-step process that involves cell cycle regulators, suppressor genes, oncogenes and their receptors, apoptosis, angiogenesis and immune surveillance (Braicu at al. 2009). A better understanding of the molecular mechanisms in hepatocarcinogenesis could identify new biomarkers for early detection and with prognostic significance for HCC, and moreover new targeted molecular therapies. The purpose of a targeted therapy is to inactivate the oncogenes, recover tumor suppressor genes, or to repair genes and molecules associated with HCC progress, in groups of patients with similar genetic and molecular anomalies.

The tumor-suppressor gene p53, named the "guardian of genome", holds an essential role in preserving stability by preventing genome mutation (Abusail et al. 2013). The major function of the gene is blockage of cell cycle progression in response to DNA damage (Xu et al., 2012). Loss of p53 function in cancers occurs mostly due to allelic deletions at chromosome 17p13 (Jain et al., 2010). Mutant forms of p53 have a prolonged halflife, which favors intranuclear accumulation, becoming detectable immunohistochemically (Zhou et al., 2006). In several studies, the overexpression of p53 in the serum or liver tissues of HCC patients was associated with a poorer prognosis, a shorter survival time and tumor recurrence (Caruso et al., 1999; Hsia et al., 2000; Qin et al., 2001; Liu et al., 2012). In our research, overexpression of p53 was found in 44% of HCCs. Our study showed that p53 overexpression was significantly associated with the poor histological differentiation of the tumor cells (p=0.008), which is consistent with previous reports (Ito et al, 2001; Qin et al., 2001; Lee et al., 2002; Sung et al., 2005).

EGFR (ErbB1/HER1) is a member of the ErbB family of receptor tyrosine kinases that plays an important role in tumor progression. Overexpression of EGFR was found in several human cancers, including HCC (Fan et al. 2014). In previous studies, overexpression of EGFR in HCC, varies from 3% to 85% (Nakopoulou et al., 1994; Yamaguchi et al., 1995; Altimari et al., 2003; Daveau et al., 2003; Hua et al., 2011). In addition, several studies found that overexpression of EGFR is correlated with early tumor recurrence and a poor prognostic in high grade HCCs (Yamaguchi et al., 1995; Daveau et al., 2003). In this study, we demonstrated that EGFR is overexpressed in 38% of HCCs, which is consistent with Bassullu N et al. study (2012). Also, we have found a significant correlation between EGFR overexpression and the histologic grade (p=0.025), suggesting that EGFR has an important role in malignant transformation of the hepatocytes.

EFGR inhibitors are promising therapeutic targets (Ito et al., 2001; Rampone et al., 2009; Berasain et al., 2011). Several EGFR agents such cetuximab panitumumab, gefitinib and erlotinib gained U.S. Food and Drug Administration (FDA) approval in recent years for different cancers (Bassullu et al., 2012).

In HCC, the response to EGFR inhibitors is not clear. In vitro, examination of EGFR inhibitors effects on HCC cells lines, demonstrated that they induce cell cycle arrest, apoptosis and increased the sensitivity to chemotherapy (Ito et al., 2001). Some studies conclude that the anti-EGFR agents have no effective response in patients with HCC (Bonner et al., 2010), while other have shown therapeutic effects in patients in a phase II clinical trial (Heesue et al., 2011). However, targeted agents are currently being evaluated in preclinical and clinical trials and the results are awaited with great interest.

Despite numerous studies on the topic of EGFR in HCC, little is known about the relation between EGFR and p53. In the present study, we have found a significant correlation between EGFR intensity and p53 overexpression (p=0.014). The study conducted by Huang et al. (2011) analyzed the mechanism of resistance to EGFR inhibitors in lung cancer and demonstrated that p53 plays an important role in regulating acquired resistance to EGFR inhibitors and radiation. It seems that loss of p53 affects response to EGFR inhibitors and radiation via regulation of cell-cycle arrest, apoptosis, and DNA damage repair (Zender et al., 2008). On the other hand, Oden-Gangloff et al. (2009) studied the resistance of anti-EGFR treatment in colorectal carcinoma and they concluded that anti-EGFR activation is oncogenic only if TP53 is inactivated, therefore the patients with TP53 mutations may be more sensitive to EGFR inhibitors. However, further large scale studies, *in vivo* and *in vitro*, are necessary to understand the relation between EGFR and p53 in HCC and their impact on prognosis and targeted therapy.

In conclusion, a better understanding of the molecular and genetic mechanisms in HCC can promote the development of targeted therapies for patients with HCC. Our results suggests that overexpression of p53 and EGFR plays an important role in hepatocarcinogenesis and contributes to more advanced disease. In correlation, these markers are not only valuable predictors of prognosis in HCC, but they are also rational targets for new anti-tumor strategies.

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