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Original Article

Downregulation of P-cadherin expression in hepatocellular carcinoma induces tumorigenicity

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Abstract: P-cadherin is a major contributor to cell-cell adhesion in epithelial tissues, playing pivotal roles in important morphogenetic and differentiation processes and in maintaining tissue integrity and homeostasis. Alterations of P-cadherin expression have been observed during the progression of several carcinomas where it appears to act as tumor suppressive or oncogenic in a context-dependent manner. Here, we found a significant downregulation of P-cadherin in hepatocellular carcinoma (HCC) cell lines and tissues compared to primary human hepatocytes and non-malignant liver tissues. Combined immunohistochemical analysis of a tissue microarray containing matched pairs of HCC tissue and corresponding non-tumorous liver tissue of 69 patients confirmed reduced P-cadherin expression in more than half of the cases. In 35 human HCC tissues, the P-cadherin immunosignal was completely lost which correlated with tumor staging and proliferation. Also *in vitro,* P-cadherin suppression in HCC cells *via* siRNA induced proliferation compared to cells transfected with control-siRNA. In summary, downregulation of P-cadherin expression appears to induce tumorigenicity in HCC. Therefore, P-cadherin expression may serve as a prognostic marker and therapeutic target of this highly aggressive tumor.

Keywords: P-cadherin, hepatocellular carcinoma, tumor staging, proliferation

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of cancer mortality [1, 2]. Highest occurrence rates are reported for Eastern and Southeastern Asia but the incidence of HCC is increasing in Western countries [3]. Morbidity and mortality correlate directly with surgical resectability of the primary tumor. However, outcome is mostly poor, since most patients are diagnosed at an advanced stage, and only 10-20% of HCCs can be resected completely [4, 5]. Hence, limited treatment options and the poor prognosis of HCC emphasize the importance of developing therapeutics as well as novel diagnostics for early detection of this highly aggressive tumor.

Cadherins belong to an important family of glycosylated Ca^{2+}-dependent adhesion molecules [6]. They are comprised of a large extracellular domain responsible for homophilic cell-cell interactions, a transmembrane domain and a highly conserved cytoplasmatic tail that is bridged to the actin cytoskeleton by binding directly and indirectly to various cytoplasmatic proteins [7]. Cadherins are localized to adherens junctions, participate in the maintenance of cell-cell contacts and control diverse morphogenetic events. In pathological processes, they play a dominant role in tumor development and progression [8]. The most widely distributed members of the cadherin superfamily are the classical cadherins. Best studied protein members of this cadherin subfamily are E-cadherin and N-cadherin, and their aberrant expression has been shown to affect the development and progression of several types of cancers including HCC [9].

Also P-cadherin (CDH3) is one of the classical cadherins. However, unlike the E- and N-ca-

Table 1. P-Cadherin immunoreactivity (IR) in HCC-tissue of 69 patients in relation to clinico-pathological characteristics and proliferation rate

Variable				P-Cad IR	P-Cad IR	*P**
	Categorization	n	%	negative	positive	
Clinico-pathological characteristics						
Age at diagnosis						
	< 60 years	31	*44.9*	13	18	0.314
	≥ 60 years	38	*55.1*	11	27	
Gender						
	female	13	*18.8*	7	6	0.194
	male	56	*81.2*	17	39	
Tumor stage						
	pT1	14	*20.3*	2	12	0.024
	pT2	22	*31.9*	11	11	
	pT3	31	*44.9*	9	22	
	pT4	2	*2.9*	2	0	
Histological grade						
	G1	23	*33.3*	8	15	0.667
	G2	38	*55.1*	12	26	
	G3	8	*11.6*	4	4	
Tumor size						
	≤ 5 cm	31	*44.9*	10	21	0.768
	> 5 cm	22	*31.9*	6	16	
	nd	16	*23.2*			
Genesis						
	alcoholic	30	*43.5*	8	22	0.358
	viral hepatitis (HBV/HCV)	13	18.8	4	9	
	others/unknown	26	*37.7*	12	14	
Proliferation rate (MIB1-Index)						
	≤ 10%	24	*34.8*	7	17	0.001
	> 10%	45	*65.2*	32	13	

*Fisher's exact test (2-sided); bold face representing *P*-values < 0.05. (nd, no data available; IR, immunoreactivity).

dherins, P-cadherin has not been fully examined at the molecular level and its physiological and pathological roles remain unclear. A recent study shows P-cadherin with a proposed unique set of interactions during formation of an intermediate X-dimer suggesting important biological implications on the specific recognition mediated by human P-cadherin [10]. P-cadherin was first identified in mouse placenta [11]. In humans, P-cadherin expression is not detectable in the placenta but in other organs, such as mammary gland and prostate [12]. Furthermore, existing data show that truncation, misexpression or dyslocalization of P-cadherin play a role in several types of cancers. However, this adhesion molecule seems to exert divergent effects depending in the type of tumor and the stage in which the tumor is, respectively [13-15]. In oral squamous cell carcinoma, malignant melanoma and bladder cancer reduction and truncation of P-cadherin expression in late stages increase tumor invasiveness and the risk of metastasis [13, 15, 16]. Moreover, decreased P-cadherin expression and cytoplasmatic localization correlate with poor clinical outcome and reduced survival rates in these three tumor entities [13, 16, 17]. Also in Merkel cell carcinoma and gastric carcinoma, P-cadherin expression indicates favorable prognosis [18, 19]. In contrast, P-cadherin overexpression seems to induce the progresion of breast and colon cancer [20, 21].

The aim of the present study was to investigate the expression and function of P-cadherin in HCC.

Materials and methods

Cells and cell culture

The HCC cell lines Huh7 (ATCC PTA-4583), HepG2 (ATCC HB-8065), PLC (ATCC CRL-8024) and Hep3B (ATCC HB-8064) were cultured as described. Primary human hepatocytes (PHH) were isolated and cultured as described [22].

Human tissues and HCC tissue microarray

Paired HCC and non-neoplastic liver tissues were obtained from HCC patients undergoing surgical resection. Tissue samples were immediately snap-frozen and stored at -80°C until subsequent analysis. A tissue microarray (TMA) of paraffin-embedded HCC samples was constructed as described [23]. Clinicopathological patient characteristics are summarized in **Table 1**.

Human liver tissue for cell isolation and expression analyses was obtained from patients with

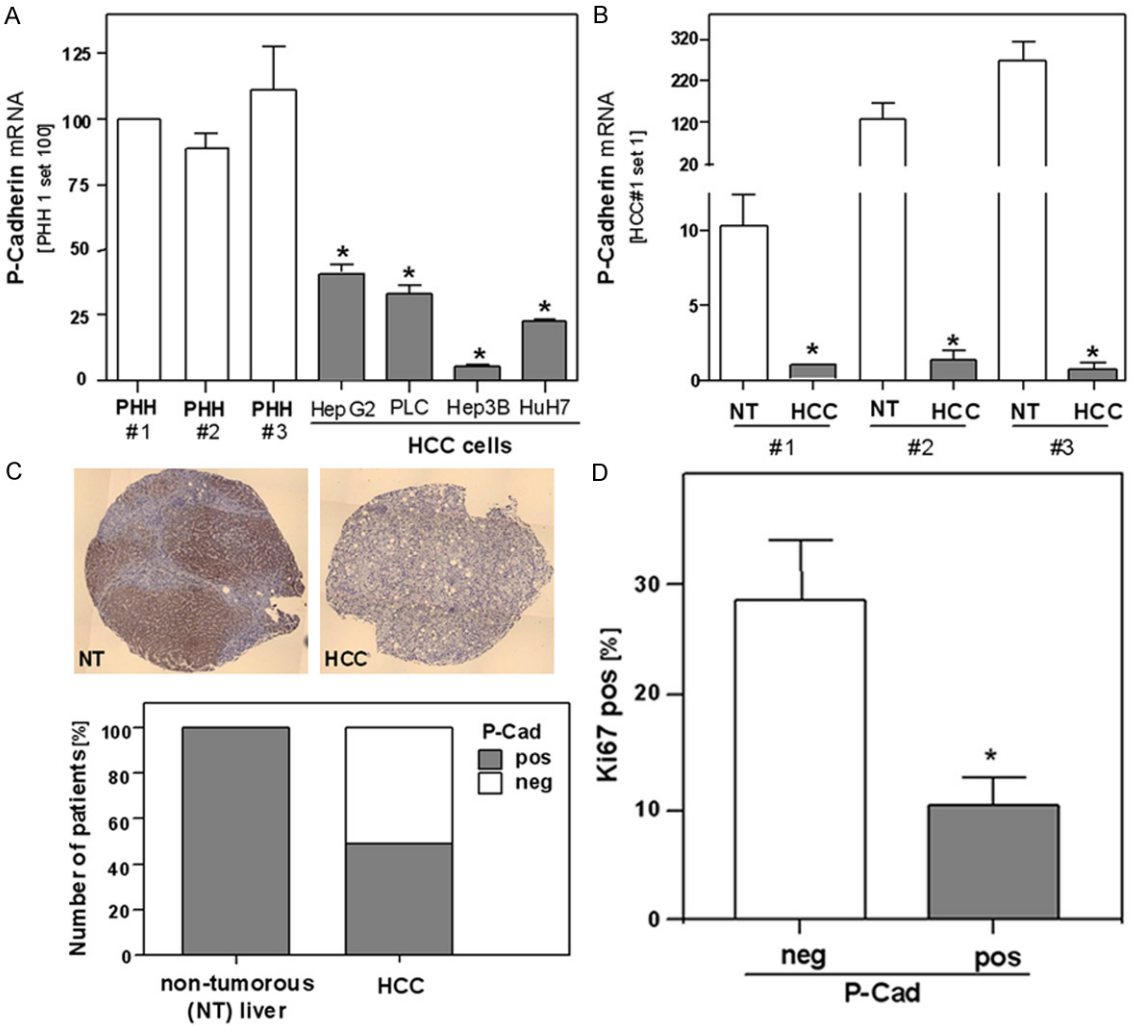

Figure 1. P-cadherin expression in HCC cells and tissues. A. Quantitative realtime RT-PCR of P-cadherin expression in primary human hepatocytes (PHH) of three different donors and four HCC cell lines (HepG2, PLC, Huh7 and Hep3B) (*: $P < 0.05$ compared to PHH); B. P-cadherin expression in human HCC tissues and corresponding non-tumorous (NT) liver tissue (*: $P < 0.05$ compared to NT); C. Representative images of strong cytoplasmatic P-cadherin staining in non-tumorous tissue and missing P-cadherin expression in corresponding HCC tissue (upper panel). Percentage of tissues with and without detectable P-cadherin immunosignal (lower panel); D. Percentage of Ki67-positice cells in HCC tissues with and without positive P-cadherin immunosignal (*: $P < 0.05$).

informed consent through the Grosshadern Tissue Bank after partial hepatectomy. The tissue bank is regulated according to the guidelines of the non-profit state-controlled HTCR (Human Tissue and Cell Research) foundation following study approval [24].

Transient transfection of small interfering RNA-P-cadherin

HepG2 cells were transiently transfected with small interfering RNA (siRNA) specific against human P-cadherin using the XtremeGene HP DNA transfection reagent (Roche, Penzberg, Germany) and Dharmafect-1 (Thermo Scientific, Bonn, Germany) according to the manufacturer's instructions. Briefly, cells were seeded the day before transfection in medium without antibiotics in 6-well cell culture plates. Subsequently, the cells were transfected with 2 µg siRNA against P-cadherin and control siRNA, respectively.

Analysis of RNA expression

Total cellular RNA from cells and tissues was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription of 1 µg RNA to complementary DNA (cDNA) was per-

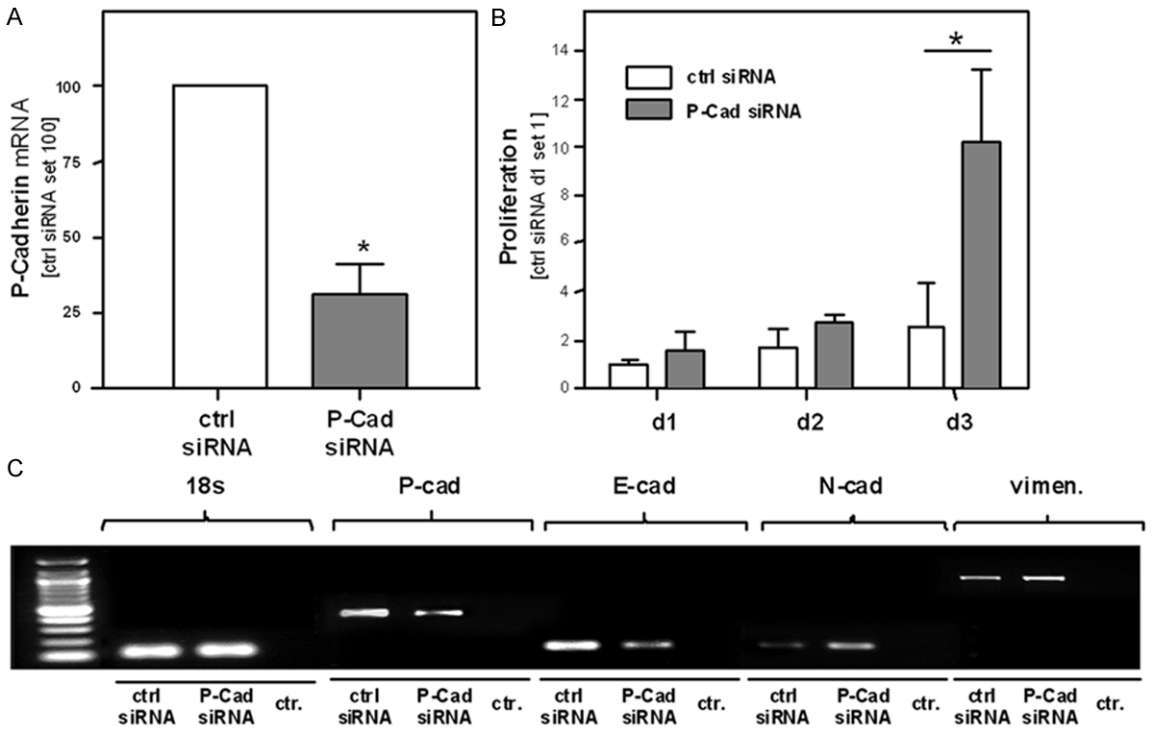

Figure 2. Inhibition of P-cadherin expression in HCC cells with siRNA *in vitro*. A. Quantitative realtime RT-PCR analysis of P-cadherin expression in HCC cells transiently transfected with siRNA directed against P-cadherin (Pcad-siRNA) or control cells transfected with scrambled RNA (ctrl siRNA) (*: $P < 0.05$); B. Analysis of proliferation of HCC cells with and without P-cadherin via siRNA over time (day 1, day 2 and 3) using a WST proliferation assay (*: $P < 0.05$); C. Analysis of E-cadherin, N-cadherin and vimentin expression in HCC cells transfected with siRNA directed against P-cadherin (Pcad-siRNA) compared to control cells transfected with scrambled siRNA (ctrl siRNA). qPCR analysis without cDNA (ctr) was performed as negative control.

formed using transcriptor high fidelity cDNA synthesis kit (Roche) according to the manufacturer's protocol. cDNA was amplified using the Stratagene Brilliant III ultra fast quantitative polymerase chain reaction master mix (Agilent Technologies, Santa Clara, CA, USA) in combination with TaqMan UPL probes (Roche). Realtime PCR primers were obtained from TibMolBiol (Berlin, Germany). 18 s messenger RNA (mRNA) was used for normalization. 18 s: 5'-gcaattattccccatgaacg-3' and 5'-gggacttaatcaacgcaagc-3', probe #48. P-cadherin: 5'-gctgggaaagtattcatgg-3' and 5'-ccttcagtgaccttcttcctg-3, probe #61.

Semiquantitative RT-PCR was performed as described previously using P-cadherin primers without probes [14]. E-cadherin: 5'-taatttttaggttagagggttattgt-3' and 5'-cacaaccaatcaacaacaca-3', N-cadherin: 5'-ccagagtttactgccatgacg-3' and 5'-tctccgccactgattctgta-3', vimentin: 5'-atgtccaccaggtccg-3' and 5'-ttattcaaggtcatcgtgat-3'.

Analysis of P-cadherin protein expression

Immunohistochemical staining of 5 µm sections of the TMA blocks was performed using mouse anti-P-cadherin antibody (1:1,000, BD Biosciences, Heidelberg, Germany) and an indirect immunoperoxidase protocol according to the LSAB2-kit (DAKO, Hamburg, Germany). A surgical pathologist (A.H.) performed a blinded evaluation of the stained slides. For negative control, the primary antibody was omitted and IgG isotype control antibodies did not reveal any detectable staining. For analysis of the tissue microarray, positivity for P-cadherin was defined as any detectable staining, cases designated as P-cadherin negative were devoid of any immunohistochemical staining for P-cadherin.

Analysis of cell proliferation

Cell proliferation was measured *in vitro* using the WST assay (Invitrogen, Life Technologies, Darmstadt, Germany) as described [15]. MIB1

expression in tissues was analyzed by immunohistochemical staining with a Ki-67 antibody as described [23].

Statistical analysis

Results are expressed as mean±standard error (range) or percent. Comparison between groups was made using the Student's unpaired t-test. A P value < 0.05 was considered statistically significant. All calculations were performed by using the GraphPad Prism Software (GraphPad Software, Inc., San Diego, USA) or SPSS (SPSS, Chicago, IL, USA). Contingency table analysis and the two-sided Fisher's exact test were used to study the statistical association between clinicopathological and immunohistochemical variables.

Results

P-cadherin expression in HCC

P-cadherin mRNA expression was analyzed in four different HCC cell lines (HepG2, PLC, Huh7 and Hep3B) and primary human hepatocytes (PHH) of three different donors by quantitative real-time PCR. In all HCC cell lines P-cadherin mRNA expression was significantly decreased compared to PHHs (**Figure 1A**). Comparison of human HCC tissues and corresponding non-tumorous liver tissues confirmed the marked downregulation of P-cadherin in cancerous tissue (**Figure 1B**). To further evaluate P-cadherin expression in a larger cohort of HCC patients, we performed P-cadherin immunohistochemical staining of a tissue microarray (TMA) comprising HCC and corresponding non-tumorous liver tissues of 85 patients [23, 25, 26]. P-cadherin immunohistochemistry (IH) was informative in 69 HCC tissues, and in 56 of these cases also in corresponding non-tumorous liver tissues. In 35 of 69 HCCs (50.7%) the P-cadherin immunosignal was completely lost while P-cadherin expression was detectable in all 57 non-tumorous liver tissue samples (**Figure 1C**, lower panel). In matched samples, IH analysis revealed reduced P-cadherin expression in 33 of 56 (58.9%) HCCs compared to non-tumorous liver tissues. In the upper panel of **Figure 1C** representative images of strong cytoplasmatic P-cadherin staining in non-tumorous tissue and missing P-cadherin expression in corresponding HCC tissue are depicted.

P-cadherin correlates with HCC tumor staging and proliferation

For descriptive data analysis, HCCs were separated into tissues with and without P-cadherin immunosignal, and immunohistochemical results were correlated with clinicopathological tumor characteristics (**Table 1**). Loss of P-cadherin significantly correlated with tumor staging (P=0.024) (**Table 1**). Moreover, we observed a highly significant correlation between reduced P-cadherin expression and the MIB1-labeling index in HCC tumor samples (P=0.001) (**Table 1** and **Figure 1D**). No correlation was found between the intensity of the P-Cadherin immunosignal and age, gender, histological grading, tumor or the presence of cirrhosis.

Inhibition of P-cadherin expression in HCC cells leads to proliferation and upregulation of EMT markers

To gain insight into the functional role of P-cadherin in HCC, we inhibited P-cadherin expression in HepG2 cells *via* transient transfection with siRNA directed against P-cadherin. HepG2 cells transfected with non-specific control siRNA served as control (ctrl.). We chose HepG2 cells since they exhibited the highest basal P-cadherin expression compared to the other tested HCC cell lines (**Figure 1A**). Quantitative realtime RT-PCR revealed a strong downregulation of P-cadherin expression with siRNA against P-cadherin compared cells transfected with control siRNA (**Figure 2A**). To characterize the role of P-cadherin in HCC cells, we performed *in vitro* WST proliferation assays with P-cadherin suppressed HCC cells in comparison to control cells. HCC cells with reduced P-cadherin expression outperformed control cells 3 times after 3 days of growth (**Figure 2B**). These data were in line with TMA data presented in **Table 1**, which showed a significant correlation of P-cadherin reduction and proliferative activity. Additionally, we tested the impact of P-cadherin knockdown on EMT marker expression in HCC cells. After 48 hours of siRNA transfection P-cadherin knockdown resulted in a downregulation of E-cadherin and upregulation of N-cadherin and vimentin (**Figure 2C**) indicative for EMT-transition in response to P-cadherin suppression.

Discussion

There are contrasting studies about the role of the classical cadherin P-cadherin in human cancers pointing toward a dual role of P-cadherin as both an oncogene as well as a tumor suppressor [27, 28]. In this study, we found reduced expression of P-cadherin in HCC-cell lines and tissues. Functional *in vitro* analysis of P-cadherin suppressed HCC cells and correlation of P-cadherin expression with clinicopathological characteristics in clinical samples showed that loss of P-cadherin negatively regulates proliferation. Moreover, loss of P-cadherin expression significantly correlated with the histological tumor staging. Both proliferation and staging are significantly related to tumor recurrence and patient survival. Recently, we have shown that P-cadherin is linked to the regulation of differentiation and cell proliferation in oral keratinocytes and oral squamous cell carcinoma [15]. Moreover, previous studies demonstrated the role of other classical cadherins in signal transduction pathway regulating cell proliferation. Thus, E-cadherin negatively regulates divergent classes of receptor tyrosine kinases, by inhibiting their ligand-dependent activation, and E-cadherin neutralizing antibodies inhibited proliferation of epithelial cell lines [29]. Also N-cadherin has been shown to negatively regulate proliferation of osteoblasts *via* inhibition of autocrine/paracrine Wnt3a ligand expression and attenuation of Wnt, ERK and PI3K/Akt signaling [30]. In contrast, previous functional studies with cholangiocarcinoma, pancreas or ovarial cancer cell lines found no effects of P-cadherin expression on cell proliferation [31, 32, 33] Together, these findings further underscore that the effects of aberrant P-cadherin expression are tumor specific.

So far, we can only speculate on the underlying molecular mechanisms by which loss of P-cadherin affects HCC cells. P-cadherin seems to induce translocation processes of proteins associated with mesenchymal to epithelial transition (MET) such as Snail *via* the GSK3-beta axis [34]. Complementary but fitting to this, we observed that downregulation of P-cadherin in HCC cells enhanced N-cadherin but lead to a downregulation of E-cadherin expression, indicative for epithelial to mesenchymal transition (EMT). Moreover, we have previously shown that P-cadherin interacts context specific with the Slit/Robo-signaling system to control migration in oral squamous cell carcinoma cells [35]. Furthermore, cadherins have been shown to play roles in signal transduction of growth factors [29, 36, 37]. For example, E-cadherin is associated with epidermal growth factor receptor (EGFR), thus activating the mitogen-activated protein kinase pathway. N-cadherin has also been found to interact with fibroblast growth factor receptors. Furthermore, P-cadherin interacts with insulin-like growth factor receptor (IGFR) [31]. MET, Slit/Robo-signaling and IGFRs have been shown to critically affect hepatocancerogenesis [38-40]. Future studies have to unravel whether P-cadherin effects in HCC are mediated *via* these or so far unidentified mechanisms. The data presented in this study can contribute to the development of new diagnostic markers and therapeutic targets for HCC.

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Disclosure of conflict of interest

None.

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