Correlation of MicroRNA-16, MicroRNA-21 and MicroRNA-101 Expression with Cyclooxygenase-2 Expression and Angiogenic Factors in Cirrhotic and Noncirrhotic Human Hepatocellular Carcinoma

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Abstract

Background: Hepatocellular carcinoma (HCC) is a classical example of inflammation-linked cancer and is characterized by hypervascularity suggesting rich angiogenesis. Cyclooxygenase-2 (COX-2) is a potent mediator of inflammation and is considered to upregulate angiogenesis. The aims of the study are (1) to analyze expression of Cox-2 mRNA, Cox-2 protein, miR-16, miR-21 and miR-101 in HCC and adjacent liver parenchyma in cirrhotic and noncirrhotic liver, (2) to investigate the relation between COX-2 expression, miR-21 expression and angiogenic factors in these tissues and (3) to investigate the association between miR-16 and miR-101 and COX-2 expression.

Methods: Tissue samples of HCC and adjacent liver parenchyma of 21 noncirrhotic livers and 20 cirrhotic livers were analyzed for COX-2 expression at the mRNA level (qRT-PCR) and at the protein level by Western blot and immunohistochemistry. Gene expression of VEGFA, VEGFR1, VEGFR2, Ang-1, Ang-2 and Tie-2 were correlated with COX-2 levels. miR-16, miR-21 and miR-101 gene expression levels were quantified in HCC tumor tissue.

Results: COX-2 mRNA and protein levels were lower in HCC as compared to adjacent liver parenchyma both in cirrhotic and noncirrhotic liver. COX-2 protein localized mainly in vascular and sinusoidal endothelial cells and in Kupffer cells. At the mRNA level but not at the protein level, COX-2 correlated with mRNA levels of angiogenic factors VEGFR1, Ang-1, and Tie2. miR-21 expression was higher in cirrhotic versus noncirrhotic adjacent liver parenchyma. None of the miRNAs correlated with COX-2 expression. miR-21 correlated negatively with Tie-2 receptor in adjacent liver parenchyma.

Conclusions: In human HCC, COX-2 mRNA but not COX-2 protein levels are associated with expression levels of angiogenic factors. MiR-21 levels are not associated with angiogenic molecules. MiR-16 and miR-101 levels do not correlate with COX-2 mRNA and protein levels.

Introduction

More than 80% of hepatocellular carcinomas (HCC) are associated with chronic infection caused by hepatitis B or C virus [1]. In well-developed countries with a high incidence of obesity and diabetes mellitus the risk of developing HCC rises proportionally with increasing body mass index and the duration of diabetes [2,3]. These conditions are frequently associated with non-alcoholic steatohepatitis (NASH) [4,5]. The common denominator in viral hepatitis associated liver diseases and NASH is presumed to be the chronic inflammation leading to fibrosis and cirrhosis and ultimately to HCC. HCC is thus one of the classical examples of inflammation-linked cancer [6]. The cyclooxygenase-2 (COX-2)-prostanoid pathway plays a pivotal role in inflammation and in the pathophysiology of liver diseases like cirrhosis and HCC [7]. COX-2 is an inducible immediate-early gene originally found to be induced by various stimuli including mitogens, cytokines and growth factors [8]. COX-2 might favor tumor growth by various mechanisms including stimulation of angiogen-
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Samples from histological normal livers (n = 8), obtained from surplus donor liver or partial liver resection for benign disorders, were used as a reference and not for statistical comparisons. HCCs were graded according to the Edmonson classification and COX-2 mRNA level and protein expression was compared among the three classes.

RNA extraction and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Quantitative RT-PCR was performed as described previously [21]. Intron overlapping primer sets and minor groove binder probes were purchased as Assay-on-Demand from Applied Biosystems (Nieuwekerk a/d IJssel, The Netherlands): housekeeping gene GAPDH (assay ID Hs99999905_m1), COX-2 (assay ID Hs00133133_m1), VEGF (Hs01176326_m1), VEGFR-1 (Hs00176573_m1), VEGFR-2 (Hs00176676_m1), Tie2 (assay ID Hs00176696_m1), Ang-1 (assay ID Hs00181613_m1), Ang-2 (assay ID Hs00169867_m1).

MiRNA analysis

Expression levels of miR-16, miR-21 and miR-101 were determined by qRT-PCR using RNU49 as housekeeping gene with miRNA qRT-PCR assays (Applied Biosystems, Foster City, USA) as described previously [22]. Reverse transcription (RT) primers specific for a miRNA and RNU49 were multiplexed in 15 µl RT reactions containing 1 µl of each RT primer. The miRNA levels were normalized to the RNU49 levels. Mean cycle threshold (Ct) values for all genes were quantified with the SDS software (version 2.1). Relative expression levels were calculated as 2^-ΔΔCt.

Western blot

Western blotting was performed as described previously using mouse anti-COX-2, 1:500 (BD Biosciences Pharmingen, #610204) [21]. Quantification of the signals detected was done by relating COX-2 to β-actin control. The bands of COX-2 protein located at about 70 kDa as expected according to the datasheet of the antibody.

Immunohistochemical examination of COX-2 expression

Four µm tissue sections were deparaffinized and treated with 0.05% H2O2 for 30 min to block endogenous peroxidase. Slides were incubated with mouse anti-COX-2 (BD Biosciences Pharmingen, #610204) 1:50, diluted in 1%BSA/PBS, at 4°C overnight followed by incubation with horseradish peroxidase-conjugated rabbit anti-mouse Ig and goat anti-rabbit Ig (DAKO), both 1:100 diluted in 1%BSA/1% albumin/PBS. Diaminobenzidin was used to develop the staining reaction and nuclear counterstaining was performed with haematoxylin. A sample of a human colon adenocarcinoma was included as a positive control.

Correlation between COX-2 protein and mRNA level

Pearson correlation coefficients were calculated for COX-2 mRNA and COX-2 protein separately for HCC and liver parenchyma. We calculated the COX-2 protein:COX-2 mRNA ratio for each individual case; this method has been used for identification of the strength of correlation of miRNA expression and protein level of individual genes by Pradet et al [23].

Correlation of COX-2 and miR-21 with Ang-2:Ang1 ratio

Although in many tumors both Ang-1 and Ang-2 are elevated, the angiogenic state of hypervascular tumors is characterized by
an overexpression of Ang-2 in relation to Ang-1 thus creating a shift in the Ang-2:Ang-1 ratio in favor of Ang-2 [24]. A higher Ang2:Ang1 ratio was also encountered in human HCC [24]. Therefore we analyzed the correlation between COX-2, miR-21 and the Ang-2:Ang-1 gene expression ratio.

Statistical analysis
Quantitative data were expressed as median and interquartile range (IQR). Expression of COX-2 mRNA, COX-2 protein, miR-16, miR-21 and miR-101 were compared using a two-tailed Mann-Whitney-U test (non-related samples) in cirrhotic versus noncirrhotic liver of HCC and adjacent parenchyma separately. The Wilcoxon test (related samples) was used for comparison of HCC with adjacent parenchyma in cirrhotic and noncirrhotic liver separately. For correlation statistics, values were normalised by log (base 10) transformation. Pearson correlation coefficients were calculated between COX-2 and the angiogenic factors, between miR-21 and the angiogenic factors and between miR-101 and miR-16 and both COX-2 mRNA and COX-2 protein. A p-value <0.05 was considered significant.

Results
COX-2 expression in HCC and adjacent liver parenchyma in cirrhotic and noncirrhotic liver
HCC tumor tissue in both cirrhotic and noncirrhotic liver demonstrated a lower COX-2 expression compared to adjacent parenchyma, both at the mRNA (Figure 1A) and protein level (Figure 1B). Median COX-2 mRNA level in the tumor in noncirrhotic liver was about tenfold lower than in tumor-adjacent liver parenchyma (p < 0.001). In cirrhotic liver, HCC tumor had a six-fold lower COX-2 mRNA content than adjacent parenchyma (p < 0.001). Protein levels of COX-2 were approximately 370 fold lower in HCC in noncirrhotic liver as compared to adjacent liver parenchyma (p = 0.033). In cirrhotic liver, HCC tumor had a 30% lower COX-2 protein level (p = 0.020) than adjacent parenchyma. A comparison between COX-2 mRNA and COX-2 protein expression in cirrhotic versus noncirrhotic HCC tumor tissue revealed no differences (Figures 1A and 1B). COX-2 staining in HCC was mainly found in endothelial cells lining the sinusoid-like spaces in the tumor but less conspicuous than in vascular endothelial cells at the interface between tumor and adjacent liver parenchyma. (Figure 2) As a positive control of COX-2 immunohistochemical staining we included a colon carcinoma sample which was strongly positive (Figure 2 insert). No difference was found in COX-2 mRNA level and protein expression between tumors with Edmonson grade 1, grade 2 or grade 3 (p>0.35).

COX-2 mRNA and COX-2 protein expression in tumor-adjacent liver parenchyma was comparable in cirrhotic and in noncirrhotic liver and in the same range as normal liver parenchyma (Figures 1A and 1B). COX-2 staining was observed in both vascular and sinusoidal endothelial cells as well as in Kupffer cells in normal liver and in parenchyma adjacent to HCC. No expression was found in hepatocytes or in cholangiocytes (Figure 2).

Although median COX-2 expression in HCC was less than in the surrounding parenchyma, individual cases demonstrated variable results as can also be seen in representative Western blots of COX-2 (Figure 3).

Correlation of COX-2 gene expression and protein level with angiogenic factors in HCC tumor tissue
Of the six analysed angiogenic factors, VEGFR1, Ang-1 and Tie-2 revealed a significant correlation with COX-2 mRNA levels in HCC tumor tissue both in noncirrhotic and cirrhotic liver (Table 2). Additionally, a correlation between COX-2 mRNA and VEGFR2 mRNA was observed in HCC in noncirrhotic liver only (r = 0.78, p<0.001). No significant correlations were obtained between the angiogenic factors and COX-2 protein in HCC tumor tissue. Moreover, also the Ang-2:Ang-1 gene expression ratio did not show any correlation with COX-2 expression (Table 2).

Correlation of COX-2 gene expression and protein level with angiogenic factors in adjacent liver parenchyma
We found no significant correlation between COX-2 mRNA levels and any of the angiogenic factors in tumor-adjacent liver parenchyma, except for Ang-1 in adjacent cirrhotic liver (Table 2). None of the mRNA levels of the angiogenic factors in adjacent parenchyma correlated significantly with COX-2 protein.
Figure 1. Plots representing relative expression of COX-2 mRNA (A), COX-2 protein (B), miR-16 (C), miR-21 (D) and miR-101 (E) in normal liver, HCC tumor tissue (T) and tumor-adjacent parenchyma (Ad) in noncirrhotic and cirrhotic liver. Median values and 25th–75th percentiles are shown. P-values: *<0.05; **<0.01; ***<0.001.

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Correlation of COX-2 mRNA expression and COX-2 protein level

No correlation was found between COX-2 mRNA expression and COX-2 protein level neither in HCC tumor tissue nor in adjacent parenchyma \( (p > 0.13) \). Calculation of the COX-2 protein:COX-2 mRNA ratio revealed a range from <1 to >35,000 in HCC tumor tissue and from 100 to >7,000 in liver parenchyma. These results suggest that post-transcriptional mechanisms are responsible for the highly variable COX-2 protein levels.

**Figure 2.** COX-2 immunostaining on HCC (upper left, marked with *) and adjacent liver parenchyma (lower right, marked with @). COX-2 expression is present on endothelial cells (arrows) at the interface between HCC and non-tumorous liver parenchyma. In the liver parenchyma COX-2 expression is mainly present in Kupffer cells and some inflammatory cells. Note the absence of staining in hepatocytes and tumor cells. The insert (lower right hand corner) shows COX-2 immunostaining on colon carcinoma as a positive control. doi:10.1371/journal.pone.0095826.g002

**Correlation of COX-2 mRNA expression and COX-2 protein level**

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**MiR-16 expression in HCC and liver parenchyma and correlation with COX-2 expression**

Figure 1C shows the relative expression of miR-16 in normal liver, tumor adjacent liver and in cirrhotic and noncirrhotic HCC tumor tissue. No statistically significant differences were found between any of these tissues. Also no correlation between miR-16 expression and either COX-2 mRNA or COX-2 protein was found.

**MiR-21 expression in HCC and liver parenchyma and correlation with angiogenic factors**

Relative expression levels of miR-21 were higher in cirrhotic tissues –both in HCC tumor tissue and adjacent liver parenchyma- as compared to HCC and parenchyma in non-cirrhotic tissues (Figure 1D). Furthermore, HCC in cirrhotic liver revealed a higher expression of miR-21 versus adjacent liver parenchyma. Analyzing the correlation between miR-21 expression and the angiogenic factors revealed no correlations except for a negative correlation \( (r = -0.76, p = 0.046) \) between miR-21 expression and the Tie-2 receptor in adjacent cirrhotic liver parenchyma.

**MiR-101 expression in HCC and liver parenchyma and correlation with Cox-2 expression**

Relative expression levels of miR-101 were lower in adjacent liver parenchyma in cirrhotic liver as compared to adjacent parenchyma in non-cirrhotic liver (Figure 1E). MiR-101 expression did not correlate with COX-2 expression neither at the mRNA level nor at the protein level.

**Discussion**

In this study we analyzed the expression of COX-2, miR-101 and miR-16 -both with COX-2 inhibiting effects- and miR-21 -with pro-angiogenic and pro-inflammatory effects- in relation to expression of various angiogenic factors in human HCC in cirrhotic and noncirrhotic liver. The chronic inflammation in cirrhotic liver provides a prosperous environment for development of HCC and because of the interaction between inflammation and angiogenesis we analysed the above mentioned molecules in two groups of patients; those with HCC in a cirrhotic liver and in patients with HCC in a noncirrhotic liver. The main finding of this study is that although at mRNA level COX-2 expression correlates positively with mRNA expression of the Ang-1/Tie-2 axis in both cirrhotic and noncirrhotic HCC, no such a correlation was encountered at the COX-2 protein level. As a possible explanation for this discrepancy we investigated the role of miR-101 and miR-16 in HCC tumor tissue and adjacent parenchyma. We found that

**Figure 3.** Example of representative cases analysed in this study showing Western blot of COX-2 in matched pairs of 11 noncirrhotic (upper panel) and 8 cirrhotic (lower panel) samples of HCC and tumor-adjacent liver parenchyma. (Adj: tumor-adjacent liver parenchyma; T: tumor (HCC)). doi:10.1371/journal.pone.0095826.g003
angiogenic growth factors [29,30]. MiR-21 is rather ubiquitous in turnover, microvessel density as well as expression of various cirrhotic versus noncirrhotic livers with respect to endothelial cell molecular characteristics, which is in accordance with previous despite a different parenchymal background—share several only. This adds support to the idea that clinically overt HCC—
correlations with the studied angiogenic factors, except for a
differential expression, however, did not translate into significant
compared to noncirrhotic adjacent liver parenchyma. This
liver parenchyma showed a higher miR-21 expression as
expression in HCC in cirrhotic liver and also adjacent cirrhotic
encountered in miR-21 expression. MiR-21 revealed the highest
analysed in our study, the most prominent differences were
inhibition of tumor growth and invasiveness [28]. Of the miRNAs
was associated with a less aggressive phenotype via
human cancer [12,13]. In prostate cancer cell lines it was demonstrated that miR-101
inhibits COX-2 by translational repression via binding to the 3
untranslated region (UTR) of COX-2 mRNA [25]. In colon
cancer cell lines a discrepancy was encountered between COX-2 protein levels and COX-2 mRNA expression; of six miRNAs examined only miR-101 demonstrated an inverse relation with the COX-2 protein/mRNA ratio in these cell lines [18]. The same authors investigated human primary colorectal cancer specimens and liver metastases and found the same inverse relation. In a series of 30 patients with gastric cancer more advanced tumor stages were associated with lower miR-101 expression which was inversely related to COX-2 mRNA expression [19]. All these data provide support for a possible role of miR-101 as a negative regulator of COX-2 protein expression and therefore we analyzed this interaction also in the HCC tumor tissue. However, we could not corroborate these findings since our data showed no inverse correlation between COX-2 and miR-101 in HCC.

MiR-21 is an important oncomir in many cancers and also in
HCC tumor [26,27]. MiR-21 plays a role in angiogenesis as was demonstrated in experiments with cell lines as well as in human cancer [12,13]. In prostate cancer cell lines inhibition of miR-21 was associated with a less aggressive phenotype via inhibition of tumor growth and invasiveness [28]. Of the miRNAs analysed in our study, the most prominent differences were encountered in miR-21 expression. MiR-21 revealed the highest expression in HCC in cirrhotic liver and also adjacent cirrhotic liver parenchyma showed a higher miR-21 expression as compared to noncirrhotic adjacent liver parenchyma. This differential expression, however, did not translate into significant correlations with the studied angiogenic factors, except for a negative correlation with the Tie-2 receptor in liver parenchyma only. This adds support to the idea that clinically overt HCC—despite a different parenchymal background—share several molecular characteristics, which is in accordance with previous findings in which no differences were found between HCC in cirrhotic versus noncirrhotic livers with respect to endothelial cell turnover, microvessel density as well as expression of various angiogenic growth factors [29,30]. MiR-21 is rather ubiquitous in
health and disease [14,31]. Therefore it is not surprising that
upregulation of miR-21 has a pivotal role in many inflammatory processes, like in patients with chronic liver diseases in whom miR-21 serum levels correlated with the severity of liver fibrosis and necroinflammation [17,32].

We found a discordance between COX-2 mRNA and protein level. As a possible explanation for this discrepancy post-transcriptional mechanisms, like transcript decay and translation rate, but also post-translational modifications which determine protein turnover and thereby protein expression levels have been described [33][34]. In humans highly discrepant results were observed between mRNA and protein expression in a study of human lung adenocarcinomas [35]. Thus, mRNA abundance does not necessarily translate into the detection of the corresponding protein with the currently available techniques and conclusions should be drawn cautiously [23].

The lack of correlation between miR-16, miR-101 and COX-2 expression is in contrast to findings in other tumor types like gastric and colon cancer. We propose two possible explanations for this discrepancy, (1) insufficient expression level of the miRNAs and (2) (tumor)tissue specificity of the miRNAs. It is likely that the expression of miRNAs should be above a—as yet unknown—minimum threshold level before they can exert their function [36,37]. Differences in mRNA expression might thus explain differences in functional activity. In addition, the overall abundance of all mRNA specific target genes in a given cell also may affect the targeting efficiency. For the analysis at the RNA level total tissue is used, and there might be a discrepancy between cells expressing the miRNA and the COX-2 gene. The current dogma describes that COX-1 is constitutively expressed and COX-2 is only expressed as a result of induction by a wide range of stimuli [38]. In an autopsy study on trauma victims, this concept was seriously questioned, because several healthy tissues, including the normal liver, were shown to express COX-2 [39]. We also found COX-2 expression in normal liver parenchyma; this was comparable to COX-2 expression in liver parenchyma adjacent to HCCs. In parenchyma adjacent to HCC we found that COX-2 expression was even higher than in HCC itself. Our data are in agreement with findings of Morinaga et al. who found a 2.5-fold higher COX-2 mRNA expression in adjacent liver parenchyma as

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Pearson r (r) and p-value (p) are presented in tumor and adjacent cirrhotic and noncirrhotic liver separately. Statistically significant correlations are shown in bold. Abbreviations: Ang-1/2: angiopoietin-1/2; Tie-2: tyrosine kinase with immunoglobulin-like and EGF-like domains-2; COX-2: Cyclooxygenase-2; miRNA: messenger ribonucleic acid; VEGFA: vascular endothelial growth factor A, VEGFR1/R2: vascular endothelial growth factor receptor 1/2; Tu: tumor; Adj: adjacent.
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compared to HCC [40]. In their study COX-2 protein expression -determined by immunohistochemistry- seemed to parallel miRNA expression and the authors explained this by inflammatory activity in tumor-adjacent parenchyma.

A limitation of our study is that the investigated tumor samples were obtained from clinically overt HCCs and the resulting signature will be rather different from the signature in dysplastic nodules and small -clinically undetectable- HCCs. For instance, COX-2 expression was found to be highest in dysplastic nodules with lower expression levels in more poorly differentiated advanced tumors in human HCC [41]. Another limitation of the study is the relatively low number of included patient samples.

From this study in human HCCs we conclude that COX-2 is expressed to a similar extent in cirrhotic and noncirrhotic adjacent liver parenchyma. Also no differences were found between HCC in cirrhotic and noncirrhotic liver despite the fact that COX-2 mRNA and COX-2 protein was lower in HCC in comparison to adjacent parenchyma. COX-2 mRNA levels both in cirrhotic and noncirrhotic HCC tumor tissue correlated with angiogenic factors. The inverse correlation between miR-101 and COX-2 protein –as found in colorectal cancer by others- was not found in our HCC tumor samples. The described prominent role of miR-21 as a proangiogenic miRNA could not be substantiated in this study. The clinical usefulness of COX-2 inhibitors especially in the setting of chemoprevention for the development of human HCC can only be based on randomized clinical trials since the precise regulatory mechanisms of COX-2 are far from elucidated.

Author Contributions
Conceived and designed the experiments: ASHG AvdB GM KPJJ. Performed the experiments: WZ SH ASHG. Analyzed the data: WZ AvdB ASHG GM KPJJ. Contributed reagents/materials/analysis tools: ASHG GM AvdB. Wrote the paper: WZ KPJJ.

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