Human collagen XV is a prominent histopathological component of sinusoidal capillarization in the hepatocellular carcinogenesis

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Abstract

Background Currently, the increased expression of collagen XV has been reported through the hepatocellular carcinogenesis process in mice. The aim of this study was to confirm the previous murine findings in human hepatocellular carcinoma (HCC) specimen along with histopathological distribution of collagen XV in tumoral tissues.

Methods Sixty-three primary HCC specimens were examined. Immunostaining of collagen XV and quantitative reverse transcriptional PCR of *COL15A1* which encodes collagen XV were performed.

Results Positive staining of collagen XV was observed in all tumoral regions regardless of differentiated level or pathological type of HCC; along with the sinusoid-like endothelium, though collagen XV was not expressed in any non-tumoral region. The intensity score of collagen XV-immunostaining and mRNA value of *COL15A1* were significantly correlated. The *COL15A1* expression in tumor elevated a 3.24-fold compared with non-tumoral regions. Multivariate analysis showed that *COL15A1* expression was significantly higher with the absence of hepatitis virus and moderately differentiated HCC.

Conclusions *COL15A1* mRNA was up-regulated in HCC and collagen XV was expressed along the sinusoid-like endothelium of HCC but not in non-tumoral region, which insinuates that collagen XV contributes to the capillarization of HCC.

Key words collagen XV, hepatocellular carcinoma, liver sinusoid, capillarization, fenestrated capillary endothelium, basement membrane

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related mortality worldwide [1, 2]. Various processes of hepatocellular carcinogenesis are considered to exist, and HCC is known to have many kinds of independent distinct onset risk factors, such as male sex [3], hepatitis C virus (HCV) infection [4,5], metabolic diseases; obesity [6], diabetes [7, 8], non-alcoholic steatohepatitis (NASH) [9, 10].

Hypervascularity is raised as one of the common important characteristics of HCC. The main constituent of nutrient blood flow of normal hepatocytes is portal perfusion, but it is replaced by arterial perfusion with the hepatocellular carcinogenesis and the growth of the HCC tumor. And neovascularisation occurs flourishingly to supply arterial perfusion inside the HCC tumor [11-13].

Sinusoid vessels exist around normal hepatocytes, which consist of fenestrated endothelium to take oxygen and nutrients from the portal perfusion into the parenchymal cells, and to transport proteins of liver derivation into the systemic circulation [14, 15]. The neo vessels grow in HCC are called sinusoid-like vessels (SLV), because they histopathologically resemble the sinusoid vessels under light microscopy but rather resemble a capillary vessel supplying the arterial perfusion into the tissue than a sinusoid vessel under electron microscopy [16-21]. The concept that sinusoid vessels change into SLVs inside of the HCC tumor is called "capillarization" and has been proposed for a long time [16]. Through the process of capillarization, pathomorphological changes, such as the disappearance of fenestration and the thickened basal membrane occur, but there are still many unknown questions of this process [17-21].

In late years, the connective tissue proteomics analysis showed the increased expression of collagen XV through the

hepatocellular carcinogenesis process in two kinds of the murine hepatocellular carcinogenic models; a platelet-derived growth factor (PDGF)-C transgenic model and a phosphoinositide 3-kinase/phosphatase and tensin homolog (PTEN) null model [22]. The aim of this study was examining collagen XV in human HCC resected specimen to confirm the previous murine findings [22]; histopathological distribution of collagen XV in tumor tissues, and expression of *COL15A1* both in the tumoral region and non-tumoral region of the resected human liver tissue accordingly.

Patients and methods

Case selection

Sixty-three primary HCC specimens, some were simultaneous or heterochronous occurrence in same patients, were obtained from 50 patients who underwent resection in our hospital between 1992 and 2010. Patients' characteristics were shown in Table 1. This research was carried out according to the principles set out in the Declaration of Helsinki 1964 and all subsequent revisions, and informed consent was obtained from each patient. This research was approved by the ethics committee of Okayama University Hospital (epidemiology 565).

Immunohistochemical analyses

The tissues were fixed with 10% neutral formalin for at least 24 hours, cut into 3-mm slices and embedded in paraffin. The paraffin embedded specimens were sliced to 5-μm thicknesses, then performed hematoxylin and eosin (H&E) staining and immunostaining. The HPA017915 rabbit monoclonal antibodies (Atlas Antibodies, Stockholm, Sweden) specifically raised against the α-chain subunit of human collagen XV, was used to delineate the distribution of collagen

XV in paraffin sections of resected specimens containing both tumor and non-tumor tissue. For H&E staining and immunostaining on tissue, 5-μm sections were deparaffinized in xylene, followed by immersion in alcohol and rehydration. Then, heat-induced epitope retrieval was performed in Target Retrieval Solution 10X Concentrate (diluted 1:10 in water) (S1699; Dako, Glostrup, Denmark). The staining procedure consisted of: blocking endogenous peroxidase activity using 0.3% H_2O_2 in methanol for 10 minutes, blocking non-specific epitopes with dried skimmed milk (diluted 1:20 in PBS) for 1 hour, incubation with the primary antibody overnight at 4°C, incubation with a Vectastain *Elite* ABC Rabbit IgG Kit (PK-6101; Vector Laboratories, Burlingame, CA, USA) according to the manufacturers' recommendation, and a subsequent reaction using an EnVision™+ Kit/HRP (DAB) DAB+ Substrate Kit (K3468; Dako). Nuclear counterstaining was performed with haematoxylin. Images were acquired using a light microscope (Olympus, models BX50 and DP73, Tokyo, Japan). Staining condition were determined for using the skeletal muscles and glomerular interstitial tissue of the kidney as the positive control. Collagen XV positive capillaries and sinusoids of hepatocellular carcinoma tissue in 10 portal areas under a high-power field (200× magnifications) were scored by a blinded pathologist (K. Ichimura).

The staining intensity was scored as "negative" (no staining), "weekly" (weakly stained), "intermediately" (moderately stained) or "strongly" (heavily stained).

The double immunofluorescence staining of collagen XV and IV were also done on paraffin-embedded tissue on 5-μm sections with the same pretreatment as for immunostaining above. These staining procedures were performed with a sequential approach: sections were, incubated with Collagenase from *Clostridium histolyticum* Type IA (C9891; Sigma-Aldrich, Saint Rouis, MO, USA) for 30 minutes, incubated with the first primary antibody, HPA017915, for

overnight at 4°C, followed by the second primary antibody, the anti-collagen IV mouse monoclonal antibody PHM-12 (prediluted, Nichirei Biosciences, Tokyo, Japan), for 1 hour at room temperature, and incubated with secondary antibodies, Cy3 sheep anti-rabbit IgG (1:100, C2306; Sigma-Aldrich) and Alexa Fluor® 488 goat anti-mouse IgG (1:500, A-11001; Molecular Probes, Life Technologies, Eugene, OR, USA). The immunofluorescence images were acquired using a confocal laser scanning microscope (ZEISS, Model LSM510, Oberkochen, Germany).

RNA analyses

Quantitative reverse transcriptional PCR (qRT-PCR) assays were performed to estimate the steady-state levels of *COL15A1* transcripts. The paraffin embedded specimens were sliced to 12-μm thicknesses and tumoral and non-tumoral regions were divided and then extracted total RNA separately. Total RNA was purified using a ReliaPrep™ FFPE Total RNA Miniprep System (Promega, Madison, WI, USA) and was subsequently amplified in a 20 μl volume using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). RNA-direct™ Realtime PCR Master Mix (Toyobo, Osaka, Japan) and TaqMan® Gene Expression Assays for *COL15A1* (Hs00266332_m1), and as control β-actin (*ACTB*) (Hs01060665_g1) (Applied Biosystems) were used according to the manufacturers' recommendations. The quantitative values were calculated based on the threshold cycle number (C_t), and the fold-change in expression was calculated using the delta-delta C_t method. [23]

Statistical analyses

For descriptive statistics, continuous variables ware analyzed using paired *t*-test, or one way analysis of variance

(ANOVA), where appropriate. Multivariate analysis were performed using multiple regression analysis. A two-tailed *p* value less than 0.05 was considered statistically significant. All statistical analyses were done using the JMP 8 software (SAS Institute inc, Cary, NC, USA).

Results

Collagen XV protein distribution in HCC specimens

Human HCC specimens were examined by immunohistochemistry using antibodies above. Positive immunostaining in the interlobular arteries served as an internal control (Fig. 1a-c). The representative results of non-tumoral region (Fig. 1d-f) and tumoral region (Fig. 1g-i) of H&E and immunohistological staining were shown. Expression of collagen XV were negative in non-tumoral region of all cases, either hepatocytes or space of Disse (Fig. 1e). But collagen XV was positively stained along with the sinusoid-like endothelium in tumoral region, regardless of differentiated level or pathological type of HCC (Fig. 1h). Next, double immunofluorescence study of collagen IV and XV were done to clarify the positional investigation, because collagen IV is known to exist at sheath of Glisson (Fig. 1c), space of Disse (Fig. 1f), and basement membrane of SLVs (Fig. 1i) [18, 24, 25]. Collagen IV, but not collagen XV existed along with sinusoid endothelium around normal hepatocytes in non-tumoral region (Fig. 1f), and collagen IV and XV co-existed along with sinusoid-like endothelium (Fig. 1i). In some part, collagen XV seems to exist closer to luminal side of siunusoid-like endothelium than collagen IV. And the positive staining of collagen XV showed a clear margin of the frontal area of tumor against non-tumoral region (Fig. 2).

Classification of collagen XV immunostaining

The staining intensity was scored as “negative” (no staining), “weekly” (weakly stained), “intermediately” (moderately stained) or “strongly” (heavily stained). Each classification indicates stained percentage of sinusoid-like endothelium, 0%, 1 - 9%, 10 - 49%, and 50% or more, respectively. In cases where differences between duplicate tissue scores were observed, the higher score was considered to be the final score. The classified results and the typical figures of each classification were presented (Table 2 and Fig. 3).

***COL15A1* mRNA expression in HCC specimens**

The qRT-PCRs were performed to quantify the expression of collagen XV and the relationship with clinicopathological factors was investigated. The *COL15A1* mRNA expression was normalized with the expression of *ACTB*, and average expression of *COL15A1* in non-tumoral region was depicted as 1. Consistent with the results of the immunohistochemical analyses, qRT-PCR assays revealed a 3.24-fold increase of *COL15A1* transcript levels in tumor relative to non-tumoral regions ($p<0.0001$). The score of collagen XV immunostaining and *COL15A1* mRNA expression level significantly correlated (Fig. 4). Furthermore correlation between *COL15A1* expression and clinicopathological features were shown in Table 3. Multivariate analysis showed that *COL15A1* expression was significantly higher with the absence of hepatitis virus ($p=0.035$) and moderately differentiated type of HCC ($p=0.048$). Though the significant difference was not provided, increased tendency in histopathological presentation of the trabecular type was also shown.

Discussion

The existance of collagen XV in the HCC was confirmed in the above-mentioned mouse models [22], but had not been yet reported about presence or absence in human HCC cases. In this study, we confirmed that collagen XV exists in human HCC tissue and that collagen XV is located along sinusoid-like endothelium. And the onset of collagen XV is considered to be tumor tissues, because of the expression of collagen XV never intersected at the margin of tumoral region and non-tumoral region where no capsule of tumor existed.

Also, we were able to confirm that the expression of *COL15A1* mRNA which encodes collagen XV specifically increased in HCC tumoral regions of resected tissues and that relative expression level of *COL15A1* mRNA significantly correlated with intensity of the collagen XV immunostaining.

Collagen XV is known to exist in systemic capillary endothelial basement membrane, peripheral nerves, smooth muscles [26-28], and is known not to exist in endothelial basement membrane of fenestrated vessels, such as the sinusoidal endothelium of the liver, the alveolus endothelium of the lung, and the glomerulus endothelium of the kidney [26-28].

In the HCC tumor, sinusoidal vessels with fenestrated endothelium is changing to un-fenestrated endothelium by capillarization [16-21] and the distribution of collagen XV of HCC is equal to the formation site of basement membrane of this sinusoid-like endothelium [18, 21]. Therefore, our results suggest that collagen XV plays an important role in the formation of basement membrane and the disappearance of fenestration of endothelium through the process of the capillarization, and to support strongly that sinusoid-like endothelium has similar character to normal capillary endothelium [16-21].

Additionally, we also obtained interesting results in qRT-PCR assay. *COL15A1* mRNA expression was observed in the all HCC cases which we examined this time. But the expression level of *COL15A1* mRNA differed depending on some kinds of clinicopathological characteristics. *COL15A1* mRNA expression level was significantly high in non-viral and moderately differentiated HCC by this study results. Our findings suggest that collagen XV may play more important roles in non-viral HCC than viral HCC. This interpretation does not contradict the previous murine models because both hepatocellular carcinogenic models [22] were non-viral models.

It is known that the ratio that HCC depends on arterial perfusion is higher in moderately and poorly differentiated tumors than well differentiated tumors [11-13]. On the other hand, construction of the SLV becomes poor in poorly differentiated tumors. So expression of *COL15A1* mRNA may be the highest in moderately differentiated HCC in this study on the average (Table 3). However, the poorly differentiated tumors need the examination in more cases for the future, because there were only three of poorly differentiated cases and one of the case showed high expression of *COL15A1* in this study (Table 1 and 3).

Also, collagen XV has been known to show antitumor effect in tumors [29]. Collagen XV at the epithelial basement membrane disappears with the progress of tumor in some kinds of carcinomas, colorectal cancer and breast cancer [30, 31]. Therefore, the increased expression of collagen XV in HCC tissue observed in our study seems to contradict these existing findings [30, 31]. However, presence of collagen XV is also confirmed in intratumoral neovascular basement membrane in these colon and breast tumors [30, 31]. So increased collagen XV expression in the HCC tumor tissue observed in this study is caused by the disparities in the collagen XV amount of adjacent non-tumor tissue; the absence of collagen XV around normal sinusoidal fenestrated endothelium and non-tumoral hepatocytes which have no

basement membrane [26-28], in contrast to much collagen XV existence by the basement membrane formation with the prosperous capillarization of HCC [11-13].

Paying attention to hypervascularity of HCC, the development of the anti-angiogenetic therapy for HCC is pushed forward [32, 33], but the effect of those therapy is unsatisfied at present [34]. This study provided the expression of collagen XV in all HCC cases, and the localization of collagen XV suggests the correlation with capillarization. But the expression intensity of collagen XV varied according to clinicopathological factors. For more effective angiogenesis inhibition to HCC, understanding the mechanism of capillarization more deeply is considered to be necessary.

In conclusion, both immunohistochemical examination of collagen XV and *COL15A1* mRNA qRT-PCR showed increased expression in tumoral region as compared with non-tumoral region of resected human HCC tissue. As for the histopathological examination by the collagen XV immunostaining, collagen XV showed specific expression along the sinusoid-like endothelium inside of HCC tumor, and collagen XV is considered to be the factor contributes to the capillarization.

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Table 1 Clinicopathological characteristics of HCC tumors

Characteristics	Tumor (n=63)
Median age, years (range)	66.2 (48–85)
Sex, n (%)	
male	58 (92.1)
female	5 (7.9)
Hepatitis viral infection, n (%)	
HCV (+)	55 (87.3)
HBV (+)	3 (4.8)
none	5 (7.9)
Differentiated level, n (%)	
well	11 (17.5)
moderately	49 (77.8)
poorly	3 (4.8)
Pathological classification, n (%)	
trabecular	54 (85.7)
others	9 (14.3)
Tumor size, n (%)	
<2cm	13 (20.6)
≧2cm	50 (79.4)

HCV; hepatitis C virus, HBV; hepatitis B virus.

Table 2 Collagen XV immunohistochemistry in SLV of tumoral region (n=63)

Stained intensity	Negative	Weakly positive	Intermediately positive	Strongly positive
n (%)	0 (0%)	13 (20.6%)	36 (57.1%)	14 (22.2%)

Negative, no SLV stained; Weakly positive, 1-9% of SLV stained; Intermediately positive, 10-49% of SLV stained; Strongly positive, 50-100% of SLV stained.

Table 3 Correlation between clinicopathological characteristics and *COL15A1* mRNA

		COL15A1 expression #	95% CI	*p* value
Sex				
	Male	3.41	2.61 - 4.44	0.473
	Female	1.79	0.68 - 4.70	
Pathological classification				
	Trabecular	3.38	2.54 – 4.50	0.104
	Others	1.98	1.47 – 4.22	
Hepatitis viral infection				
	Infected	3.02	2.33 – 3.93	0.035*
	Not infected	6.15	2.19 – 17.25	
Tumor size				
	≧2cm	3.45	2.60 – 4.58	0.463
	<2cm	2.53	1.35 – 4.75	
Differentiated level				
	Well	1.83	0.87 – 3.84	
	Moderate	3.76	2.86 – 4.95	0.048*
	Poorly	2.25	0.24 – 20.32	

P-value was calculated by multiple regression analysis.

#: The average *COL15A1* expression level in non-tumor regions is depicted as 1.

*, Statistically significant.

Figure captions

Fig. 1.

H&E staining (**a, d, g**), immunostaining of collagen XV (**b, e, h**) and fluorescence double staining of collagens IV (green) and XV (red) (**c, f, i**) of Glisson (**a-c**), non-tumoral tissue surrounding HCC (**d-f**) and HCC tissue (**g-i**) are presented. Collagen XV is expressed in the interlobular arteries (ia), but not in the interlobular veins (iv) or bile ducts (ib) at Glisson (**b, c**). Collagen XV expression is positive in all tumoral regions (**h, i**) and negative in all non-tumoral regions (**e, f**). Collagen IV is known to be expressed in Disse of normal liver (**f**) and the basement membrane of sinusoid-like endothelium of HCC (**i**). The co-existance with collagen IV shows that collagen XV also exists around sinusoid-like endothelium (**i**).

ia, interlobular artery; iv, interlobular vein; ib, interlobular bile duct; arrow, sheath of Glisson. Scale bars **a, b, d, e, g, h**, 100 μm; **c, f, i**, 40 μm.

Fig. 2.

H&E staining (**a**) and collagen XV immunostaining (**b**) at the border of the tumor front, which do not have a capsule to separate the HCC (right upper part, **a, b**) and non-cancerous liver (left lower part, **a, b**). The collagen XV stained areas remains only within the tumoral region (**b**). Scale bars equal 100 μm.

Fig. 3.

Representative figures of collagen XV immunostaining of each intensity. Each sample was classified as weakly positive (less than 10% positivity) (**a**), intermediately positive (10–49% positivity) (**b**), or strongly positive (50% or more positive) (**c**) according to the percentage of positively stained sinusoid-like endothelium. There was no negative case in this study. Scale bars equal 100 μm.

Fig. 4.

Relative mRNA expression levels of *COL15A1* in HCC and non-tumoral region (left side) and those of each classification of collagen XV immunostaining (right side). Left side, the 3.24-fold increase of *COL15A1* transcript levels in tumoral regions relative to that in non-tumoral regions is shown, on average. Paired *t*-test and one-way ANOVA was used. ★ means p=0.044. ★★★ means $p < 0.0001$.