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Expression of a secretory protein C1qTNF6, a C1qTNF family member, in hepatocellular carcinoma

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Abstract. *Background*: Recent studies have revealed that the adiponectin-associated protein belonging to the C1qTNF family mediates various biological processes. However, the pathobiological property of C1qTNF6 in carcinogenesis remains unclear. Here, we investigated the expression status of C1qTNF6 in human hepatocellular carcinomas and subsequently attempted to determine the role of C1qTNF6 in tumor neovascularization. *Methods*: Immunohistochemical staining was performed to evaluate the expression of C1qTNF6 in hepatocellular carcinoma tissue specimens. Various eukaryotic recombinant C1qTNF6 proteins were prepared to ask whether C1qTNF6 could activate Akt pathway in human liver sinusoidal microvascular endothelial cells. Xenograft assay was carried out to know the effect of C1qTNF6 on tumor neovascularization. Results: C1qTNF6 was not immunohistochemically detected in any non-cancerous liver tissues but was detected in 21 of 30 hepatocellular carcinoma tissue specimens. C1qTNF6 was not uniformly distributed but rather focally localized in hepatocellular carcinoma cells. Interestingly, it was also localized on the tumor endothelial cells, which were in close proximity of C1qTNF6-expressing hepatocellular carcinoma cells. Eukaryotic recombinant C1qTNF6 increased the level of active phosphorylated Akt molecules in cultured vascular endothelial cells via its C-terminal C1q domain. In the xenograft assay, enforced expression of C1qTNF6 markedly reduced the central hypovascular necrosis areas of the transplanted HepG2 hepatocellular carcinoma cells. *Conclusion*: These results indicate that C1qTNF6 is overexpressed and possibly contributes to tumor angiogenesis by activating the Akt pathway in many hepatocellular carcinomas.

Keywords: C1qTNF6, hepatocellular carcinoma, tumor neovascularization, Akt

1. Introduction

Tumor neovascularization is critical for the growth of malignant tumors [1, 2]. Hepatocellular carcinoma (HCC), in particular, is a hypervascular neoplasm that requires a rich blood supply for its growth; therefore, various approaches involving the interruption of blood supply have been used for the treatment of patients with HCC [3–5].

C1qTNF6 [C1qTNF-related protein 6, also designated CTRP6 (C1qTNF-related protein-6)] has a unique molecular structure, including a putative N-terminal signal peptide, short variable region, collagen-like domain, and C1q globular domain [6]. Several secretory molecules, including adiponectin [7–14], have a similar molecular structure and constitute a highly conserved protein family, the C1qTNF-related protein superfamily [15, 16]. Although crystal structure analysis indicated that the proteins belonging to the C1qTNF family originated from a common ancestral innate immunity-associated secretory protein [17], each C1qTNF family member has a distinct biological property.

For example, adiponectin, a circulating adipocyte-derived secretory C1qTNF family protein, is known to be a key regulator of insulin sensitivity [9]. C1qTNF1 inhibits collagen-induced platelet aggregation in vascular systems [10] as well as stimulates aldosterone production in the adrenal glands [11]. C1qTNF3 (also

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known as cartducin) participates in chondrogenesis as well as promotes proliferation and migration of vascular endothelial cells [12, 13]. Mutation of C1qTNF5 is responsible for the development of late-onset retinal macular degeneration with choroidal neovascularization and chorioretinal atrophy [14].

C1qTNF6 is also a C1qTNF family member [6] and is thought to play a role in fatty acid metabolism [18]. A recent study provides the evidence that C1qTNF6 induces the expression of interleukin-10 in macrophages [19] and thus may have an anti-inflammatory property. However, the expression status and pathologic role of C1qTNF6 in carcinogenesis remains unclear. Here, we report that C1qTNF6 was overexpressed in many HCCs, promoted the phosphorylation of Akt molecules in sinusoidal microvascular endothelial cells, and accelerated neovascularization in transplanted HCC cells. Controlling the level of secretory C1qTNF6 might offer a new therapeutic approach to abrogate the growth of HCC.

2. Materials and methods

2.1. Antibodies

Both anti-Flag M2 and anti-β-actin murine monoclonal antibodies were purchased from Sigma-Aldrich (Sigma-Aldrich, St Louis, MO). We used 2 commercially available specific rabbit antibodies to C1qTNF6 (raised against a recombinant protein consisting of total 133 amino acids; Sigma-Aldrich, catalog number HPA002042) and a 15-amino-acid-long peptide (available as anti-CTRP6 antibody, catalog number 54562, AnaSpec Inc., San Jose, CA). Rabbit antibodies to Akt and Akt phosphorylated at Ser473 were purchased from Cell Signaling Technology (Beverly, MA). Normal control rabbit IgG was prepared at our laboratory.

2.2. Immunohistochemical staining

Details of the procedures used for immunohistochemical staining were described previously [20, 21]. Archival pathological tissue specimens were obtained from 30 patients with HCC. All specimens were surgically obtained, fixed in 10% buffered formalin, and embedded in paraffin. The tissue specimens were immunostained with antibodies using Envision reagent (DAKO, Kyoto, Japan). In several experiments, antibodies were preadsorbed with full-length recombinant C1qTNF6, the preparation of which is described below.

2.3. Cell culture

The 293FT cells (Invitrogen, Carlsbad, CA) were maintained at our laboratory. A human hepatocellular carcinoma cell line, HepG2 cell line was obtained from the Japan Health Science Research Resources Bank (Osaka, Japan). The 293FT and HepG2 cells were cultured in Dulbecco's modified Eagle's medium (D-MEM) (Gibco Life Technologies, NY) containing 10% heat-inactivated fetal bovine serum (FBS) and 50 μg/ml gentamycin (Gibco).

Normal human liver sinusoidal microvascular endothelial cells were purchased from the Applied Cell Biology Research Institute (Kirkland, WA) and cultured in Cell Systems complete (CS-C) medium (Cell System, Kirkland, WA), as previously described [21].

2.4. Reverse transcriptase-polymerase chain reaction and plasmids

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed as previously described [21]. Briefly, total cellular RNA was prepared from cell lysates using RNA-zol B (Biotex Laboratory, Houston, TX). cDNA synthesis from total RNA and subsequent PCR were performed using an RNA LA (long and accurate) PCR kit (Takara).

The human full-length C1qTNF6 cDNA was amplified by RT-PCR using placental total RNA (Takara-Clontech, Ohtsu, Japan) and the following primers: sense, 5′-GGCCATGCAGTGGCTCAGGG TCCGTGAG-3′; antisense, 5′-TCAGTCGTCCTCGG CCTTGATGAGG-3′. Next, the cDNA was cloned into pTargeT mammalian expression vector (Promega, Madison, WI), which was designated pTatgeT-C1qTNF6. To generate a C-terminally tagged protein, the $6 \times$ His sequences were inserted immediately before the stop codon of C1qTNF6 by using an antisense primer 5′-TCAGTGGTGATGGTGATGATG GTCGTCCTCGGCCTTGATGAGG-3′ (nucleotides corresponding to the $6 \times$ His sequence have been underlined); this vector was designated human pTargeT-His-C1qTNF6.

We also prepared an expression vector, in which a Flag epitope was inserted after the putative sig-

nal sequence of human C1qTNF6 to generate an N-terminally tagged protein, Flag-C1qTNF6. Briefly, a human *C1qTNF6* nucleotide corresponding to the coding region immediately after the putative signal sequence was amplified by using PrimeSTAR GXL DNA Polymerase (Takara) and the following primers: sense, 5′-GACAAGCTTGACTACAAGGA CGACGATGACAAGGACGCGGCCCAGCCGGCC AGGCGCGCCGTA-3′ (underlined and boldface sequences indicate the *Hind*III restriction site and FLAG tag, respectively); antisense, 5′-TCAGTCG TCCTCGGCCTTGATGAGG-3′. The PCR product was cut with *Hind*III and inserted into the downstream region of the Igκ-chain leader sequence of pSecTag2 vector (Invitrogen Life Sciences, Carlsbad, CA) between the *Hind*III and *EcoR*V restriction sites.

Various 3′-terminal side-deleted C1qTNF6 cDNAs were further prepared to obtain the N-terminally FLAG-tagged truncated C1qTNF6 protein (Fig. 1) using the antisense primer 5′-TCAGAAGAAGCGC TTCTGGCAC-3′ or 5′-TCAAGCAGCAAACTGGC CGGTC-3′ instead of 5′-TCAGTCGTCCTCGGCCT TGATGAGG-3′.

The entire coding regions of plasmids were verified by sequencing using an ABI 310 autosequencer (Perkin-Elmer, Waltham, MA).

2.5. Recombinant proteins

Eukaryotic recombinant His-tagged C1qTNF6 was obtained from the culture supernatant of 293FT cells, which were transfected with pTargeT-His-C1qTNF6 by using DOTAP transfection reagent (Boehringer Mannheim GmbH, Mannheim, Germany), as described previously [22]. Culture supernatants were harvested 3 days after transfection to prepare the His-tagged C1qTNF6 by using the Ni-NTA Spin Kit (Qiagen, Valencia, CA).

The pSecTag2 expression vector containing full length and various 3′-terminal side-deleted C1qTNF6 cDNAs were also transfected to 293FT cells by using the DOTAP reagent. Three days after transfection, culture supernatant was harvested to obtain the N-terminally FLAG-tagged C1qTNF6 proteins.

2.6. Western immunoblotting

Western immunoblotting was carried out according to the modified method of Towbin et al. [23] as previously reported [24]. Briefly, equal amounts of proteins were electrophoresed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and electroblotted to polyvinylidine difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with bovine serum albumin and subsequently incubated with antibodies.

2.7. Xenografts of HepG2 cells in BALB/c nude mice

RT-PCR experiments did not detect the C1qTNF6 mRNA expression in human HCC cells, HepG2. HepG2 cells were transfected with pTargetT-C1qTNF6 or empty vector by using the DOTAP reagent. After G418 selection, 3 C1qTNF6-expressing and non-C1qTNF6-expressing HepG2 cell clones were established. The following primer sets used to detect the 615 bp of C1qTNF6 cDNA: 5′-ATGGAGA CAGACACACTCCTGC-3′ (sense) and 5′-CGAAGA AGCGCTTCTGGCAC-3′ (antisense). The sense and antisense primers used to detect glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) were 5′-TCCA CCACCCTGTTGCTGTA-3′ and 5′-ACCACAGTCC ATGCCATCAC-3′, respectively.

Cell proliferation was evaluated by determining the number of live cells, as previously described [20]. Briefly, 1.2×10^4 HepG2 cells were cultured on standard 35-mm tissue culture dishes (BD Falcon, San Jose, CA) in triplicate. After 24 and 48 hours of culture, live cells were counted.

C1qTNF6-expressing or control HepG2 cells (1×10^7) were subcutaneously injected into the dorsal flank of 8-week-old BALB/c nude mice (Charles River Laboratories, Sizuoka, Japan). After 2 weeks of inoculation, the xenografts in nude mice were excised and subjected to formalin-fixation and paraffin-embedded tissue sectioning for histopathological analysis. The length and width of tumors were measured using a caliper with a precision of 0.5 mm. Tumor volume was calculated using the formula: volume = $(d_1 \times d_2 \times d_3) \times 0.5236$, where d_n represents the 3 orthogonal diameter measurements. The experimental protocol was approved by the Animal Care Committee of Kochi Medical School, Kochi, Japan.

Statistical analysis was performed using Student's *t*-test for unpaired observation. Values with $p < 0.01$ were considered significant.

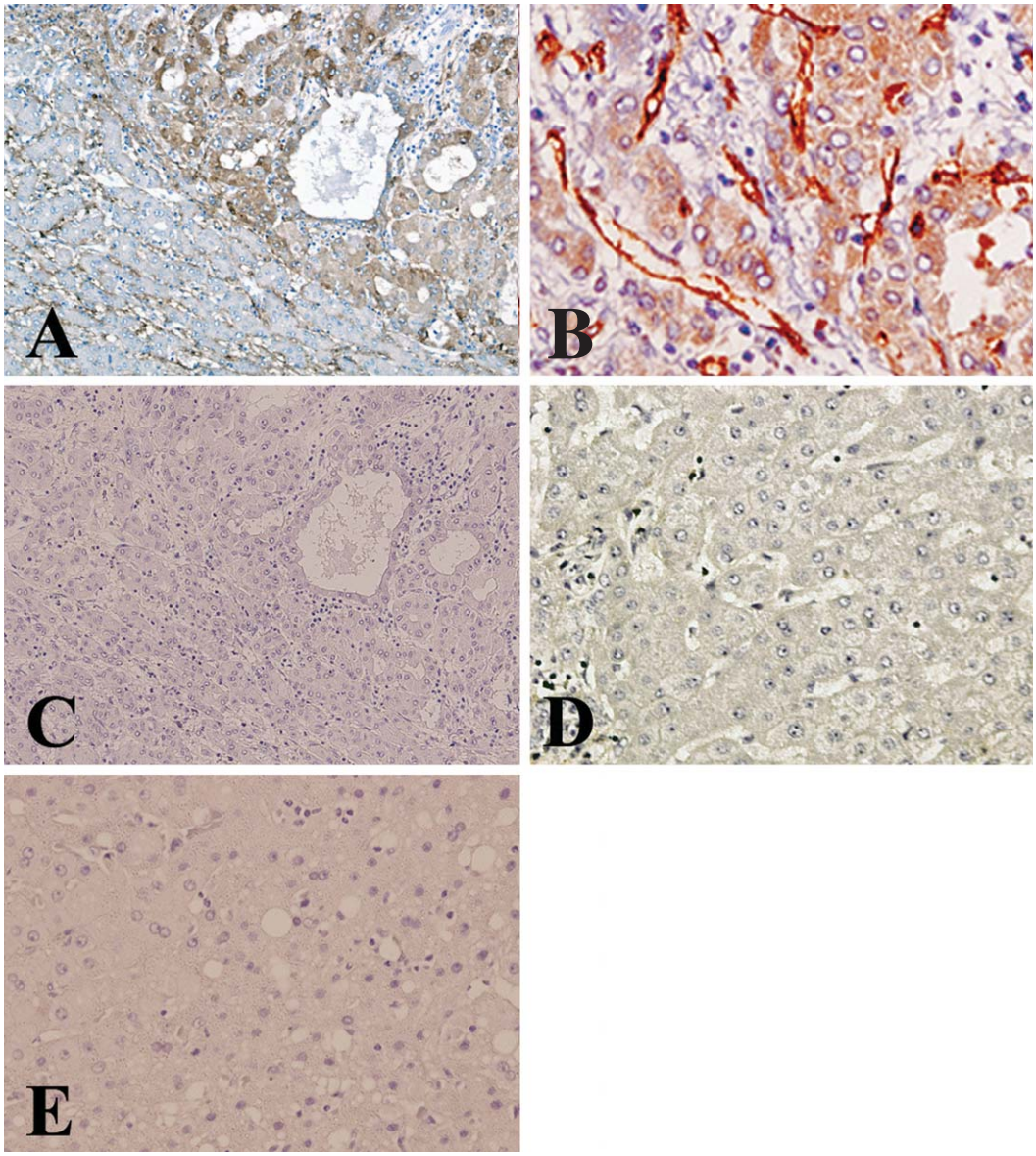

Fig. 1. Representative results of immunohistochemical staining. Immunoreactivity with anti-C1qTNF6 antibody (Sigma-Aldrich) was found in hepatocellular carcinoma cells of 21 of 30 tissue specimens. C1qTNF6 was often localized in the perivascular side of hepatocellular carcinoma cells (A). Notably, C1qTNF6 immunoreactivity was also found in the intratumoral endothelial cells of hepatocellular carcinoma that were in close proximity to C1qTNF6-positive HCC cells (B). Preadsorption with Ni-column-purified recombinant His-tagged C1qTNF6 completely eliminated the positive staining, confirming the specificity of the antibody (C). Immunoreactivity with anti-C1qTNF6 was minimal in non-cancerous regions (D: non-cirrhosis liver; E: liver cirrhosis).

3. Results

3.1. C1qTNF6 was expressed in HCC

While examining the expression of C1qTNF6 in various human tumors, we found that many HCC cells expressed C1qTNF6. Immunohistochemical staining showed that C1qTNF6 was expressed in 21 of the 30 HCC tissue specimens tested. Representative results of immunohistochemical staining are shown in Fig. 1. C1qTNF6 expression was not uniform and focal staining was noted in positive cases (Fig. 1A and B). In

Fig. 1A, immunoreactivity was found in both moderately differentiated HCC nodule (characterized as the pseudoglandular structure at upper right) and in the well differentiated HCC (characterized as relatively uniform thick cell plate at the lower left). In the present immunohistochemical staining, we did not observe the staining outside the tumor. C1qTNF6 immunoreactivity was found in not only the cytoplasm of HCC cells but also the sinusoidal lining cells Preadsorption of antibody with recombinant C1qTNF6 completely eliminated this immunoreactivity, confirming the specificity of the antibody (Fig. 1C). In contrast, immunoreactivity to anti-C1qTNF6 antibodies was rarely found in non-cancerous hepatocytes (Fig. 1D and E). No staining was observed in tissues stained with the control antibody.

Because C1qTNF6 has been little characterized, we used 2 commercially available antibodies against human C1qTNF6 to confirm the result of immunohistochemical staining. Each antibody yielded almost identical immunohistochemical staining result, except for the immunoreactivity with fibrous tissues surrounding the pseudonodules of cirrhotic liver specimens. The antibody purchased from AnaSpec immunostained the fibrous capsules but that purchased from Sigma-Aldrich did not.

We could not find any significant histopathological differences examined between C1qTNF6-positive and –negative HCC (supplementary Table 1).

The immunohistochemical staining analysis indicated that C1qTNF6 was overexpressed in many HCCs. C1qTNF6 immunoreactivity was not uniform even in the same tissue specimens and was often co-localized in HCC cells and juxtaposed tumor endothelial cells (Fig. 1A and B). Therefore, we speculated that C1qTNF6 might play a role in tumor angiogenesis by acting as a paracrine mediator for the interaction between HCC cells and tumor vascular endothelial cells.

3.2. *C1qTNF6 increased activated Akt expression in vascular endothelial cells*

C1qTNF1 and very recently C1qTNF3 were found to activate Akt pathway in the myotubes and liver, respectively [6, 25]. It is well established that phosphorylation of Akt activates the Akt-mTOR-p70S6K pathway, which in turn leads to embryonic vascular development and pathologic angiogenesis [26]. Li et al. reported that the expression of active phosphorylated Akt in tumor sinusoidal endothelial cells was significantly enhanced in HCCs and was associated with a venous and capsular invasion of HCC [27].

These findings led us to explore whether C1qTNF6 increased the expression of the active phosphorylated Akt in cultured vascular endothelial cells. As shown in Fig. 2, recombinant C1qTNF6 significantly increased the phosphorylation of Akt molecules in the human liver sinusoidal microvascular endothelial cells. Notably, the C1q domain in the C-terminal side of C1qTNF6 appeared to be critical for the C1qTNF6-mediated activation of Akt. The present finding suggests that C1qTNF6 may contribute to tumor angiogenesis in HCCs via activating the Akt pathway in vascular endothelial cells.

3.3. *Enforced C1qTNF6 expression promotes tumor neovascularization in vivo*

We next elucidated whether exogenous C1qTNF6 expression in HCC cells was associated with tumor neovascularization *in vitro* by performing xenograft assays. Three independent exogenously C1qTNF6-expressing HepG2 cell clones were established. All the 3 clones secreted C1qTNF6 (representative data are shown in Fig. 3A and B). There were no significant differences in cell growth between C1qTNF6-expressing and non-expressing HepG2 cells *in vitro* (Fig. 3C).

Both C1qTNF6-expressing and non-C1qTNF6-expressing HepG2 cells could induce tumor formation in BALB/c nude mice; the mean and standard deviation of tumor volume at 14 days after inoculation was 44.9 (8.52) mm^3 and 46.3 (5.59) mm^3 ($n = 5$), respectively. Although there was no significant difference in tumor volume between the 2 cells, the central hypovascular tumor necrosis areas, which were found in the center of non-C1qTNF6 expressing HepG2 cells (Fig. 3D), were highly reduced or not observed in xenografted C1qTNF6-expressing HepG2 cells (Fig. 3E).

4. Discussion

The intratumoral endothelial cells in HCC lose their peculiar hepatic phenotypes and acquire characteristics that help maintain abundant blood flow during tumor progression [28]. Various angiogenic factors, which are secreted by cancer cells, are important to recruit and sustain tumor vascular networks.

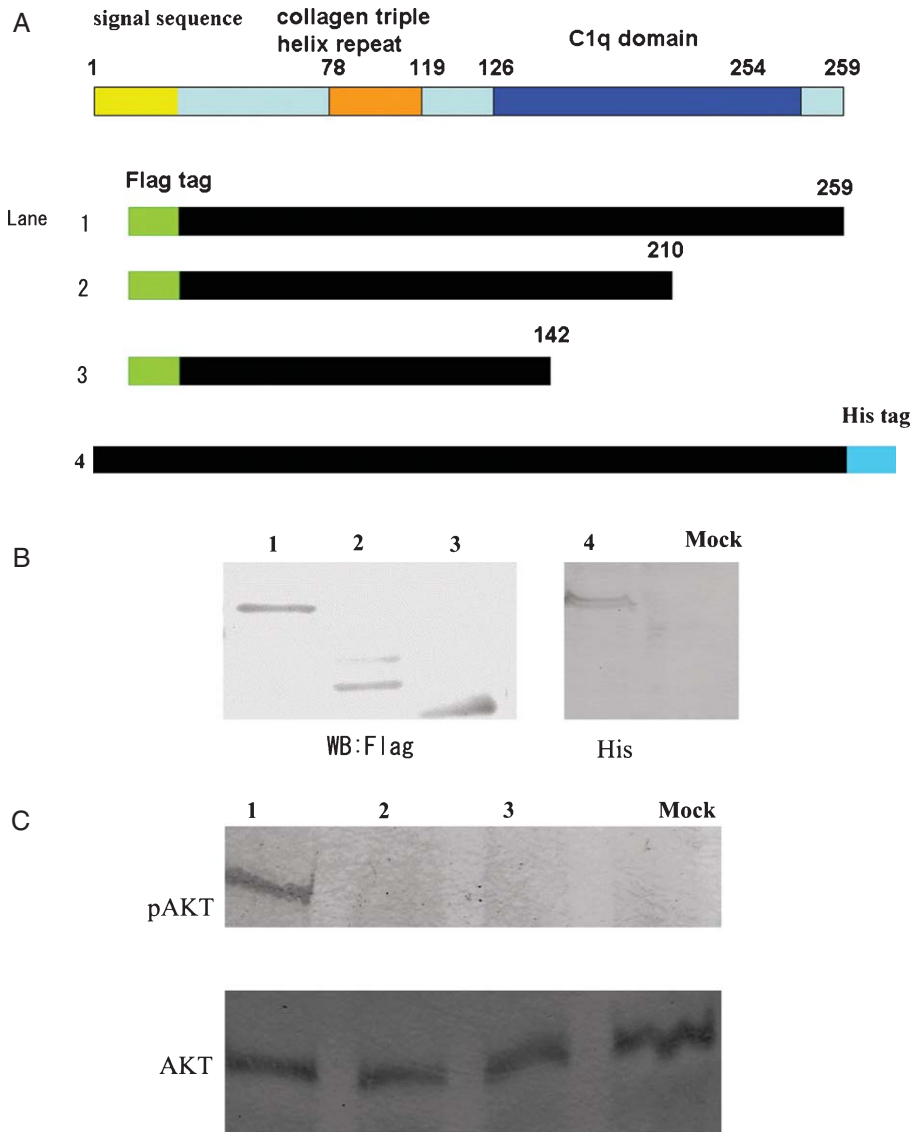

Fig. 2. C1qTNF6 increased the phosphorylation of Akt in cultured endothelial cells via the C-terminal C1q domain. A: Schematic representation of C1qTNF6 structure and recombinant proteins. B: Western immunoblotting showed that various parts of recombinant human Flag-tagged C1qTNF6 were obtained in the culture supernatant of 293FT cells using full-length (lane 1), 3′-deleted C1qTNF6-expression vectors (lane 2 and 3), or His-tagged C1qTNF6 (lane 4). C: Activation of the phosphorylation status of the C-terminal hydrophobic motif of Akt (S473 on AKT1, S474 on AKT2, and S472 on AKT3) was higher in human liver sinusoidal microvascular endothelial cells that were incubated with the culture supernatant containing full-length C1qTNF6 than in cells incubated with various C-terminal truncated C1qTNF6. Note the equal intensity of the band corresponding to the total Akt molecule in each lane.

The present study showed that C1qTNF6 was overexpressed in many HCC cells. HepG2 cell clones, which started expressing C1qTNF6 after being transfected with C1qTNF6-expression vector, secreted C1qTNF6 in the culture supernatant. Interestingly, the recombinant C1qTNF6 increased the level of active phosphorylated Akt molecules in cultured human liver sinusoidal microvascular endothelial cells. Recent studies have shown that sustained endothelial Akt activation caused increased blood vessel size and generalized edema from chronic vascular permeability [29]. The present xenograft assay also showed

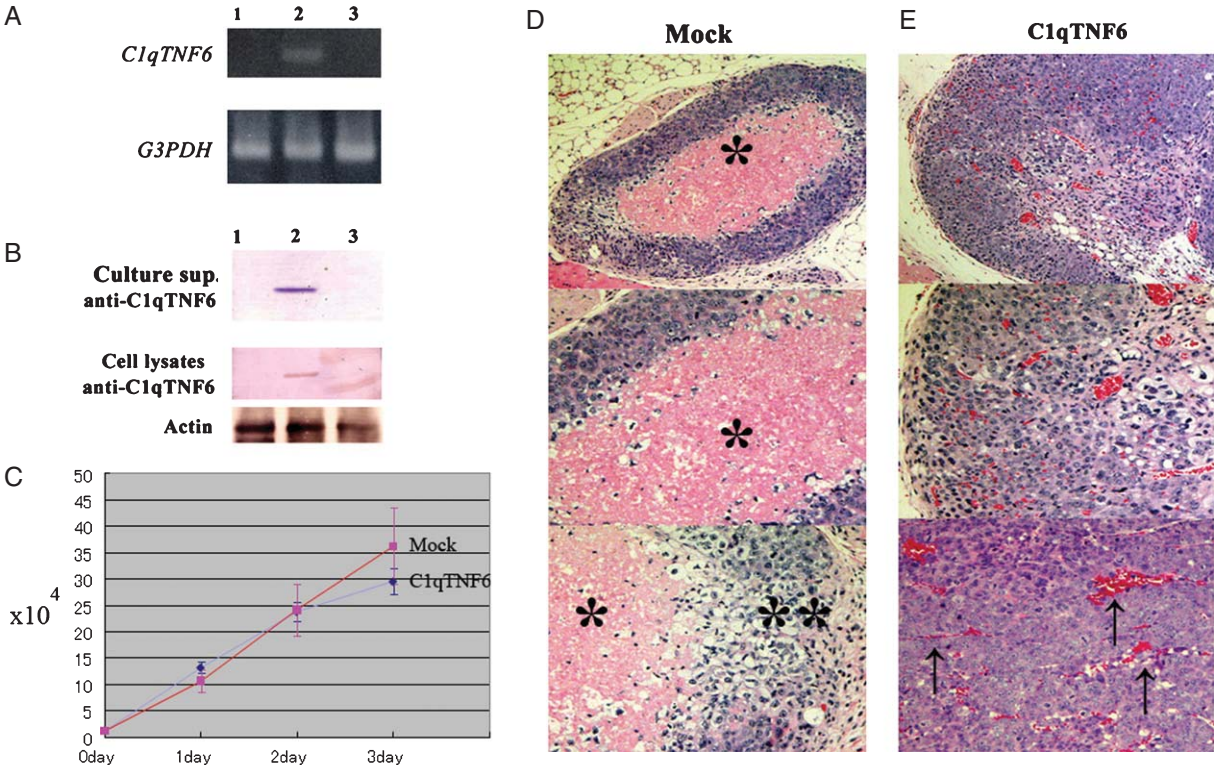

Fig. 3. C1qTNF6 expression accelerated tumor neovascularization *in vivo*. A: Representative data of RT-PCR. Original HepG2 cells did not express C1qTNF6 mRNA (lane 1). Exogenously C1qTNF6-expressing and control non-C1qTNF6 expressing HepG2 cell clones were established by transfecting the cells with an expression vector that contained full-length C1qTNF6 cDNA or with vector alone (lanes 2 and 3, respectively). A representative result from 3 independent RT-PCR assays using different transfectant clones is shown. B: C1qTNF6-expressing HepG2 cells, but not control cells, secreted C1qTNF6. A band corresponding to C1qTNF6 molecule was detected in the culture supernatant of C1qTNF6-expressing (lane 2), but not in the original (lane 1) or empty-vector transfected control HepG2 cells (lane 3) by western immunoblotting with anti-C1qTNF6 antibody. C: Exogenous C1qTNF6 expression did not affect cell growth *in vitro*. HepG2 cells were cultured on 35-mm tissue culture dishes in triplicate. After 24, 48, and 72 hours, live cells were counted. Significant differences in cell growth were found between C1qTNF6-expressing and control HepG2 cells. This was repeated 3 times using different clones and consistent results were obtained as shown in the figure. Data are expressed as mean (SD). D and E: Xenograft studies in nude mice. Representative histological features of xenograft specimens. A total of 1×10^7 cells were subcutaneously injected into nude mice. Hematoxylin-eosin-stained tissue specimens of transplanted tumors harboring non-C1qTNF6-expressing HepG2 cells (D) and C1qTNF6-expressing HepG2 cells (E) at day 14 are shown. Non-C1qTNF6-expressing cells contained large central necrosis areas [D: indicated by asterisk (*)] with little tumor-penetrating vessels. The peripheral regions consisted of altered HepG2 with clear cytoplasm [D: marked by double asterisk (**)]. In contrast, C1qTNF6-expressing HepG2 cells formed solid tumors without central necrosis and with abundant tumor-penetrating vessels (E: tumor vessels are marked by arrow). The figure represents the result from 3 independent assays, in which 3 C1qTNF6-expressing clones were used and 5 mice were injected each clone.

that C1qTNF6 expression significantly reduced the hypovascular central necrosis area in transplanted HepG2 cells. Taken together, these findings suggest that C1qTNF6 may act as an angiogenic factor, which could promote the formation of vascular network in HCC.

As shown in Fig. 1A and B, C1qTNF6 was detected on the sinusoidal lining cells in many HCC specimens. These sinusoidal lining cells are presumed but not proven to be endothelial cells. Ito cells, NK cells, and in some cases fibroblasts may also be there. However, as shown in Fig. 1B, at least a part of vascular endothelial cell was stained with specific antibody to C1qTNF6. Notably; our preliminary fluorescence-activated cell sorting (FACS) staining showed that eukaryotic recombinant C1qTNF6 was bound to the cell surface membrane of human umbilical vein endothelial cells. We speculate that a not-yet-identified

surface membrane receptor for C1qTNF6 may mediate the C1qTNF6-induced activation of Akt pathway in tumor vascular endothelial cells. Since the C1q globular domain of C1qTNF6 was crucial for the active phosphorylation of Akt in vascular endothelial cells, C1qTNF6 might bind to the receptor via its C1q domain. Interestingly, Liu et al showed that C1q itself was also able to activate the AKT pathway in dendritic cells [30]. Further study is now undertaken to identify the C1qTNF6-binding surface membrane molecule on tumor vascular endothelial cells.

In conclusion, the present study revealed the 3 unique pathobiological characteristics of C1qTNF6: it was overexpressed in many HCC cells; could activate the Akt pathway, for which the C-terminal globular domain of C1qTNF6 was important, in human liver sinusoidal microvascular endothelial cells; and contributed to angiogenesis in xenograft assay. These findings suggest that C1qTNF6 might be a therapeutically useful target for patients with HCC.

Supplementary Table 1
Relationship between expression of C1qTNF6 in HCC cells and various histopathological features

Variant	C1qTNF6 expression		
	Positive	Negative	Significance[a]
Differentiation*			
Poor	6	3	Not significant (NS)
Moderate	11	4	
Well	6	2	
Adjacent tissues to HCC			
Cirrhosis	12	5	NS
Hepatitis	9	4	
Tumor size			
>5 cm	5	0	NS
>3 cm, <5 cm	9	2	
<3 cm	7	7	
Capsule			
Present	14	6	NS
Absent	7	3	
Portal invasion			
Present	5	2	NS
Absent	16	7	
Intrahepatic metastasis			
Present	8	3	NS
Absent	13	6	
Etiology			
HBV	7	3	NS
HCV	14	6	

*In two tissue specimens, well and moderately differentiated HCC nodes were co-existed and both were stained with anti-C1qTNF6 antibody. HBV hepatitis B virus, HCV hepatitis C virus.; [a]Statistical analysis was performed using Fisher's exact test. Values with $p < 0.01$ were considered significant.

Acknowledgments

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