Concurrent expression of C4.4A and Tenascin-C in tumor cells relates to poor prognosis of esophageal squamous cell carcinoma

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Abstract. C4.4A is a glycolipid-anchored membrane protein expressed in several human malignancies. We recently found that C4.4A expression was associated with poor prognosis of esophageal squamous carcinoma cells (ESCCs), but the underlying mechanism is unknown. To uncover this, we performed PCR array analysis using the HCT116 cell line, a positive control for C4.4A expression and we found that Tenascin-C (TNC) among the many adhesion molecules and extracellular matrix proteins was the best candidate for C4.4A molecule induction. Based on *in vitro* studies using the TE8 esophageal cancer cells, we examined by immunohistochemistry TNC expression in 111 ESCCs. We found that the TNC-positive group (24.3%) had significantly poorer prognosis than the TNC-negative group in 5-year overall survival. We also found there was a significant correlation between TNC and C4.4A in ESCC tissues (P=0.007). Finally, we found that only the double-positive group for C4.4A and TNC had a significantly worse prognosis (P=0.005). Our data suggest that TNC expression in ESCC may in part explain why C4.4A is associated with a poor prognosis of ESCC since TNC can promote invasion and metastasis.

Introduction

The C4.4A protein was initially found in a metastatic rat pancreatic adenocarcinoma cell line (1,2). Rat C4.4A cDNA was cloned and the glycosylphosphatidyl inositol (GPI)-anchored membrane protein was found to have 30% homology to the urokinase-type plasminogen activator receptor (3). The human homologue of rat C4.4A, located on chromosome 19q13.1-q13.2, was subsequently cloned (4). The C4.4A mRNA is present in normal human placental tissue, skin, esophagus tissue and leukocytes (4). Although the physiological function of the C4.4A protein is largely unknown, upregulation of C4.4A expression has been observed during the wound-healing process of migrating keratinocytes and in the urothelium (5,6).

We demonstrated that C4.4A expression at the invasive front of colorectal cancer predicted disease recurrence and the C4.4A expression was associated with tumor budding and EMT change (7-9). Very recently we have shown that C4.4A expression is associated with a poor prognosis of esophageal squamous cell carcinoma (ESCC) (10). To investigate the mechanism of how C4.4A influences the prognosis of ESCC, we performed PCR-array loading in various extracellular matrix proteins and cell adhesion molecules. The result indicated that C4.4A expression correlated well with Tenascin-C (TNC) expression. TNC is an extracellular matrix protein secreted from both tumor cells and myofibroblasts. Strong expression of TNC occurs during development, starting at gastrulation. In contrast, TNC is absent or greatly reduced in most adult tissue, but it increased in some pathological conditions, inclucing inflammation, wound healing and in a variety of neoplasias. In many tumors, TNC expression correlated with invasiveness and malignancy (11-19). In this study, we attempted to examine the clinical relevance of the TNC in relation to C4.4A expression in ESCC.

Materials and methods

Clinical tissue samples. Esophageal tissue samples (n=111) were collected during surgery (1998-2007) at the Department of Surgery, Osaka University (Osaka, Japan). Chemoradiotherapy for advanced esophageal cancer is commonly used in Western countries (20,21) and neoadjuvant chemotherapy is a standard therapy before surgery in stage II and III ESCC patients in Japan (22). Therefore, we collected samples from previous ESCC patients (1998-2007) who did not receive preoperative radiotherapy and/or chemotherapy, since our institute usually employs preoperative chemotherapy using

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Table I. The molecules mounted on PCR array.

Cell adhesion molecules	
Transmembrane molecules:	CD44, CDH1, HAS1, ICAM1, ITGA1, ITGA2, ITGA3, ITGA4, ITGA5, ITGA6, ITGA7, ITGA8, ITGAL, ITGAM, ITGAV, ITGB1, ITGB2, ITGB3, ITGB4, ITGB5, MMP14, MMP15, MMP16, NCAM1, PECAM1, SELE, SELL, SELP, SGCE, SPG7, VCAM1
Cell-cell adhesion:	CD44, CDH1, COL11A1, COL14A1, COL6A2, CTNND1, ICAM1, ITGA8, VCAM1
Cell-matrix adhesion:	ADAMTS13, CD44, ITGA1, ITGA2, ITGA3, ITGA4, ITGA5, ITGA6, ITGA7, ITGA8, ITGAL, ITGAM, ITGAV, ITGB1, ITGB2, ITGB3, ITGB4, ITGB5, SGCE, SPP1, THBS3
Other adhesion molecules:	CNTN1, COL12A1, COL15A1, COL16A1, COL5A1, COL6A1, COL7A1, COL8A1, VCAN, CTGF, CTNNA1, CTNNB1, CTNND2, FN1, KAL1, LAMA1, LAMA2, LAMA3, LAMB1, LAMB3, LAMC1, THBS1, THBS2, CLEC3B, TNC, VTN
Extracellular matrix proteins	
Basement membrane constituents:	COL4A2, COL7A1, LAMA1, LAMA2, LAMA3, LAMB1, LAMB3, LAMC1, SPARC
Collagens and ECM structural: constituents	COL11A1, COL12A1, COL14A1, COL15A1, COL16A1, COL1A1, COL4A2, COL5A1, COL6A1, COL6A2, COL7A1, COL8A1, FN1, KAL1
ECM proteases:	ADAMTS1, ADAMTS13, ADAMTS8, MMP1, MMP10, MMP11, MMP12, MMP13, MMP14, MMP15, MMP16, MMP2, MMP3, MMP7, MMP8, MMP9, SPG7, TIMP1
ECM protease inhibitors:	COL7A1, KAL1, THBS1, TIMP1, TIMP2, TIMP3
Other ECM molecules:	VCAN, CTGF, ECM1, HAS1, SPP1, TGFBI, THBS2, THBS3, CLEC3B, TNC, VTN

FAP (5-FU, adriamycin, CDDP) or DCF (docetaxel, CDDP, 5-FU) in recent ESCC cases. Samples were fixed in buffered formalin at 4˚C overnight, processed through graded ethanol solutions and embedded in paraffin. A piece of tissue sample was frozen in liquid nitrogen and stored at -80˚C until protein extraction. The specimens were used appropriately and under the approval of the ethics committee at the Graduate School of Medicine, Osaka University.

Cell culture. The human colon cancer cell line HCT116 was obtained from the American Type Culture Collection (Manassas, VA, USA). The human esophageal cancer cell line TE8 was obtained from Tohoku University (Miyagi, Japan). These cells were grown in DMEM, or RPMI, respectively, supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, at 37˚C in a humidified incubator with 5% CO_2 in the air.

Collagen gel culture. Collagen gel cultures were carried out using a collagen gel kit (Nitta Gelatin, Osaka, Japan), according to the manufacturer's protocol. One milliliter of collagen solution, containing $5x10^5$ cells was overlaid on a pre-prepared basal collagen layer in a 6-well dish and incubated at 37˚C for 30 min. RPMI supplemented with 10% FBS was added after gelatinization. The culture medium was changed every day.

siRNA for C4.4A. For small interfering RNA (siRNA) inhibition, the following double-stranded RNA duplexes targeting human C4.4A were used: 5'-GCUGUAACUCUGACCUCCG CAACAA-3'/5'-UUGUUGCGGAGGUCAGAGUUACAGC-3' (siRNA I, HSS120351, Invitrogen) and 5'-CAACGUCACCU UGACGGCAGCUAAU-3'/5'-AUUAGCUGCCGUCAAGGUG ACGUUG-3' (siRNA II, HSS178302, Invitrogen). Negative-control siRNAs were purchased in a Stealth RNAi kit (Invitrogen). Cells were transfected with siRNA using Lipofectamine™ RNAiMAX (Invitrogen) according to the manufacturer's protocols.

PCR-array. Total RNA was extracted using the RNAeasy mini kit (Qiagen-Sample & Assay Technologies, Hilden, Germany). The ABI PRISM 7500 Sequence Detector (Applied Biosystems, Foster City, CA, USA) was used for PCR. RT^2PCR Array loading extracellular matrix proteins and cell adhesion molecules (code #PAHS-013A-2, Table I) were employed to analyze each sample (SABioscience, Frederick, MD, USA). For each plate, results were normalized to the median value of a set of housekeeping genes. A significant threshold of a 2-fold change in gene expression corresponded to $P<0.001$. HCT116 cell samples were prepared from the following 5 groups: i) parental cells in 2D culture for 48 h, ii) parental cells in 3D type I-collagen gel culture for 48 h, iii) 3D cultures for 48 h after 24-h treatment with C4.4A-siRNA I, iv) 3D cultures for 48 h after 24-h treatment with C4.4A-siRNA II and v) 3D cultures for 48 h after 24-h treatment with negative control-siRNA.

Quantitative real-time PCR. cDNA was generated from 1 μg total RNA using the high capacity RNA-to-cDNA kit (Applied Biosystems). Quantitative real-time PCR was carried out using the LightCycler (Idaho Technology, ID, USA) as described previously (23). Quantification data from each sample were analyzed using the LightCycler analysis software.

The C4.4A primer sequences were 5'-TCACCTTGACG GCAGCTA-3', C4.4A sense and 5'-AGCCACTGAGCGTGA ACC-3', C4.4A antisense; and the probe was UPL No. 58 (Roche). The TNC primer sequences were 5'-CTGAAGGTGG AGGGGTACAG-3', TNC sense and 5'-AGAAGGATCTGCC ATTGTGG-3', TNC antisense; and the probe was UPL No. 56

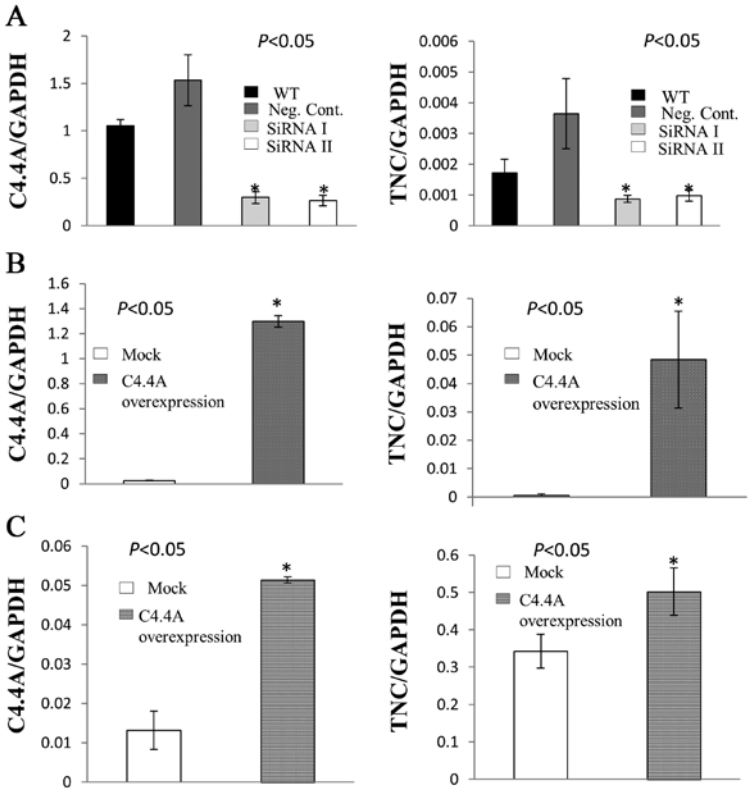

Figure 1. qRT-PCR for C4.4A mRNA and TNC mRNA from HCT116 cultures grown for 48 h in a 3D collagen matrix. (A) HCT116 cells treated with siRNA for C4.4A (24 h). (B) HCT116 cells in the lentivirus-mediated C4.4A overexpression system. (C) TE8 esophageal cancer cells in the lentivirus-mediated C4.4A overexpression system. GAPDH mRNA served as an internal control. WT, wild-type (parental cells); neg cont,-negative control; mock, empty vector transfection control with lentivirus.

(Roche). The amount of each transcript was normalized against the expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), from the same sample using the primers 5'-AGCCACATCGCTCAGACAC-3', sense and 5'-GCCCAATACGACCAAATCC-3', antisense; and the probe UPL No. 60 (Roche).

Immunohistochemistry. A rabbit anti-human C4.4A GPI-related polyclonal antibody (7-10) hereafter designated as the C4.4A-GPI antibody, was used. The anti-human TNC mouse monoclonal antibody (ab6393) was purchased from Abcam (Cambridge, UK). Tissue sections (4 μm thick) were prepared from paraffin-embedded blocks. After antigen retrieval treatment in 10 mM citrate buffer (pH 6.0) at 95˚C for 40 min, immunostaining was carried out using the Vectastain ABC peroxidase kit (Vector Laboratories, Burlingame, CA, USA), as we described previously (24,25). The slides were incubated with appropriate antibodies overnight at 4˚C at the following dilutions: C4.4A-GPI, 1:300 and TNC, 1:4,000. Non-immunized rabbit IgG or mouse IgG (Vector Laboratories) was used as a negative control and substituted for the primary antibody to exclude possible false-positive responses from the secondary antibody or from non-specific binding of IgG.

Transfection of plasmids. We stably transduced the C4.4A plasmid into HCT116 and TE8 cells with the lentivirus. Plasmids with the human C4.4A NM_014400 (Origene Inc., Rockville, MD, USA) were transfected with the pLenti6/V5 Directional TOPO® Cloning kit (Invitrogen). An empty pLenti6/V5 vector was used as a mock control.

Statistical analysis. Statistical analysis was carried out using the JMP8 program (SAS Institute, Cary, NC, USA). The Kaplan-Meier method was used to estimate tumor recurrence from CRC and the log-rank test was used to determine the statistical significance. Associations between discrete variables were assessed using the χ^2 test. Mean values were compared using the Mann-Whitney U test. P-values <0.05 were considered statistically significant.

Results

Molecular linkage of C4.4A to Tenascin-C in 3D collagen cultures. To investigate a molecular linkage of C4.4A to certain molecules, especially cell adhesion molecules and extracellular matrix proteins, we used a custom PCR array analysis (Table I). When C4.4A-positive control HCT116 cells were cultured, C4.4A mRNA levels increased 2.6-fold at 48 h in 3D cultures when compared to 2D cultures (data not shown). Thus, we searched the gene sets that were increased in 3D cultures and yet decreased when these cultures were treated with C4.4A siRNA (siRNA I, siRNA II). We then focused on TNC, which ranked at the top of the list (Table II). Confirmation studies using qRT-PCR assays showed that C4.4A siRNA treatments significantly reduced TNC mRNA

Table II. Top 15 gene lists by analysis with PCR array.

	3D:2D	siRNA I:neg cont[a]	siRNA II:neg cont[a]
TNC	3.19	0.14	0.18
HAS1	3.15	0.14	0.07
MMP13	2.54	0.33	0.44
ITGAM	2.26	1.83	0.96
ECM1	1.97	0.22	0.67
MMP16	1.88	1.1	0.87
ITGA4	1.87	0.63	0.8
MMP10	1.83	0.15	0.90
LAMA3	1.78	0.50	0.99
MMP1	1.77	1.15	0.96
COL4A2	1.73	1.87	0.42
COL12A1	1.64	0.43	0.35
NCAM1	1.52	0.76	0.96
ADAMTS1	1.51	1.22	0.07
ITGA8	1.50	0.69	0.54

[a]The ratio was calculated from data in 3-D cultures. neg cont, negative control.

in HCT116 cells in 3D cultures (Fig. 1A, P<0.05). By contrast, lentivirus-mediated forced expression of C4.4A in 3D cultures significantly enhanced TNC mRNA levels in HCT116 (Fig. 1B, P<0.05) and ESCC TE8 cells (Fig. 1C, P<0.05).

Tenascin-C expression in ESCC tissue samples. Based on the molecular linkage between C4.4A and TNC *in vitro*, we examined the TNC protein expression in tumor cells of ESCC by immunohistochemistry. We found 27 of 111 ESCCs (24.3%) expressed TNC in the cytoplasm of the tumor cells (Fig. 2). When TNC-positive cases (n=27) and TNC-negative cases (n=84) were compared for the various clinical and pathological parameters including age, sex, histology, T stage, lymphatic invasion, venous invasion, lymph node metastasis and distant metastasis, TNC expression was associated with deeper wall invasion (Table III, P=0.028).

Survival analysis by TNC expression. Survival curves were drawn between TNC-positive and TNC-negative ESCCs. The TNC-positive group had significantly poorer prognosis than the TNC-negative group in 5-year OS (Fig. 3, P=0.012). The univariate analysis indicated that TNC expression, tumor

Table III. TNC expression and clinicopathological parameters in esophagel SCC patients.

	TNC-positive (n=27)	TNC negative (n=84)	P-value
Age	38-84 (median 64.7)	48-80 (median 64.0)	NS[a]
Gender			
Male	23	77	NS
Female	4	7	
Histology[b]			
Well, Mod	24	60	NS
Por	3	24	
T classification[c]			
T1, T2	10	52	0.028
T3, T4	17	32	
Lymphatic invasion			
Positive	23	63	NS
Negative	4	21	
Venous invasion			
Positive	14	29	NS
Negative	13	55	
Lymph node metastasis			
Positive	22	53	NS
Negative	5	31	
Distant metastasis			
Positive	2	8	NS
Negative	25	76	

ESCC cases (n=111) were categorized as TNC-positive (n=27) and TNC-negative (n=84). [a]NS, not significant. [b]Well, well differenciated squamous cell carcinoma; Mod, moderately differenciated squamous cell carcinoma; por, poorly differenciated squamous cell carcinoma. [c]T1, invasiveness within submucosa; T2, invasiveness in muscularis propria; T3, invasiveness in outer membrane; T4, invasiveness over outer membrane.

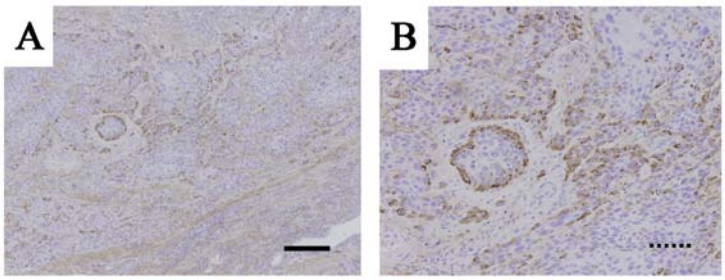

Figure 2. Immunohistochemistry for TNC in ESCC tissue samples and survival analysis. Tissue sections of ESCC were immunostained with an anti-TNC monoclonal antibody. (A) A portion of tumor cells stained positive in the cytoplasm. (B) Magnification. Tumor cells were defined as TNC-negative when no staining was observed. The black scale bar indicates 250 μm. The dot scale bar indicates 100 μm.

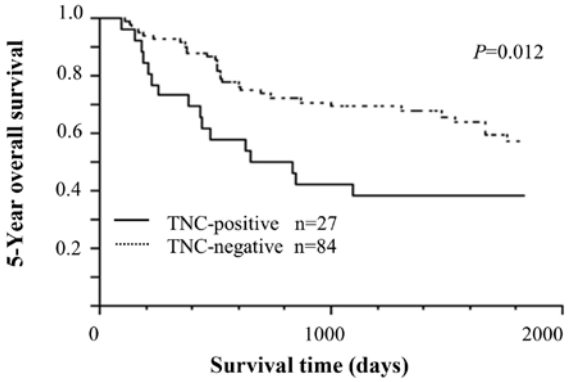

Figure 3. Survival analysis. TNC-positive group was significantly associated with poorer prognosis compared to the TNC-negative group in 5-year overall survival (P=0.012).

stage, lymph node metastasis and venous invasion were significant predictors of a poor 5-year OS (Table IV). We carried out a multivariate analysis to further determine the most significant prognostic factors. Lymph node metastasis was identified as an independent prognostic factor (P=0.045). TNC expression was not an independent prognostic factor (Table IV).

C4.4A expression in ESCC by the C4.4A-GPI antibody. C4.4A protein expression was shown in the normal epithelium of the esophagus by IHC using the C4.4A-GPI antibody. Expression was on the plasma membrane, mainly at the parabasal layer (Fig. 4A). In contrast, tumor cells in the same tissue sample did not always express the C4.4A protein (Fig. 4A). We

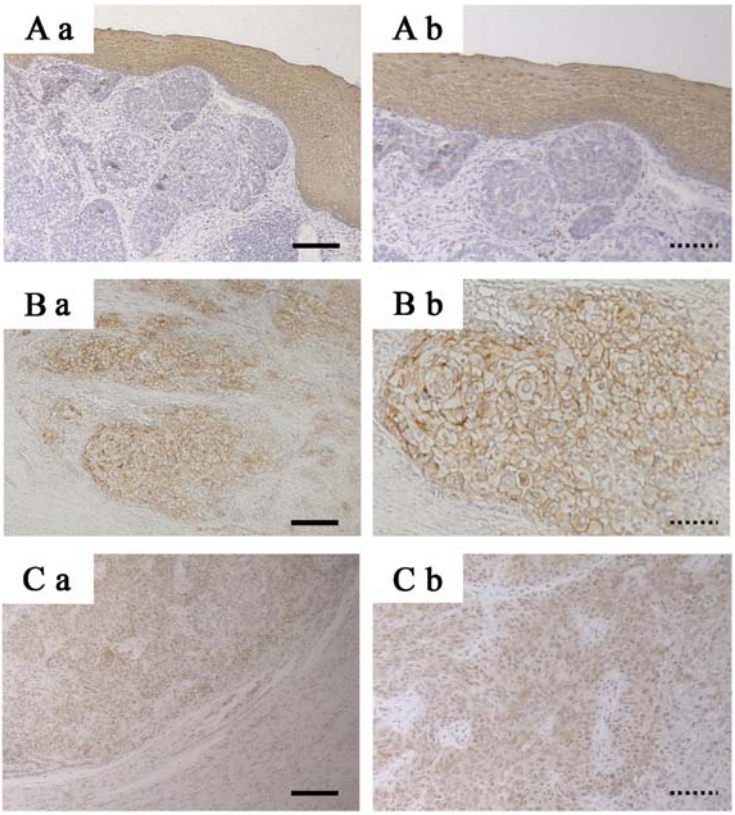

Figure 4. Immunohistochemistry for C4.4A with the anti-C4.4A-GPI antibody in ESCC tissue samples. (A) Normal esophageal squamous epithelium and a C4.4A-GPI-negative tumor. A magnified image of tumor tissue is also shown. (B) C4.4A staining on the plasma membrane and the magnified view. (C) C4.4A staining in the cytoplasm and the magnified view. The black scale bar indicates 250 μm. The dot scale bar indicates 100 μm.

Table IV. Five-year overall survival in ESCC patients (n=111).

A, Univariate analysis	Risk ratio	Confidence interval	P-value
TNC (positive vs negative)	2.139	1.137-3.876	0.019
Age (>65 vs <65 years)	1.285	0.717-2.300	NS
Sex (male vs female)	1.690	0.616-6.977	NS
T stage (T1/T2 vs T3/T4)	2.769	1.540-5.080	<0.001
Differentiation (well/mod vs por)	1.696	0.875-3.119	NS
Lymph node metastasis (positive vs negative)	3.351	1.640-7.770	<0.001
Distant metastasis (positive vs negative)	1.857	0.759-3.906	NS
Lymph invasion (positive vs negative)	2.060	0.980-5.043	NS
Venous invasion (positive vs negative)	2.104	1.175-3.787	0.013
B, Multivariate analysis	**Risk ratio**	**Confidence interval**	**P-value**
TNC (positive vs negative)	1.786	0.943-3.251	NS
T stage (T1/T2 vs T3/T4)	1.846	0.988-3.545	NS
Lymph node metastasis (positive vs negative)	2.172	1.015-5.323	0.045
Venous invasion (positive vs negative)	1.294	0.807-2.691	NS

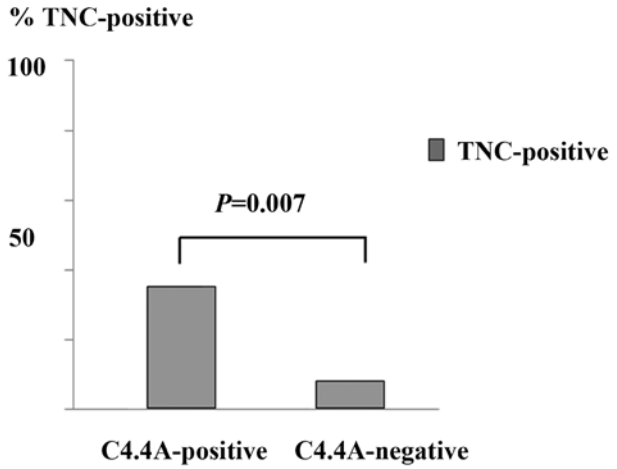

Figure 5. Association between TNC and C4.4A expression detected by the C4.4A-GPI antibody in ESCCs. A significant association was noted between TNC expression and C4.4A expression (P=0.007).

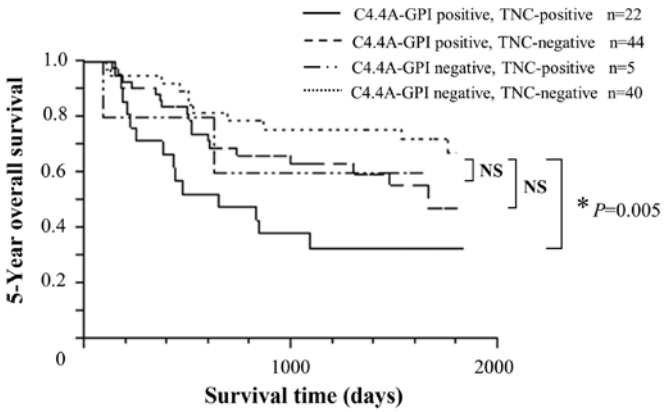

Figure 6. Sub-group analysis. There was a significant difference in OS between C4.4A-positive/TNC-positive cases and C4.4A-negative/TNC-negative cases (P=0.005). However, TNC-positive alone group or C4.4A positive alone group did not provide a statistical significance. NS, not significant.

defined the esophageal carcinoma tissues as C4.4A-negative if staining was not noted at all in the tumor cells on the tissue sections. There were 45 C4.4A-negative esophageal tumors in the 111 cases tested (40.5%); while, 66 esophageal tumors (59.5%) provided clear C4.4A staining on the plasma membrane (Fig. 4B) or cytoplasm (Fig. 4C). Sixty-six positive cases were classified according to intracellular localization, i.e., 29 membrane staining alone, 11 cytoplasmic staining alone and 26 both membrane and cytoplasmic staining. When expression of C4.4A and TNC was compared, TNC expression was significantly associated with C4.4A expression (Fig. 5, P=0.007).

Furthermore, sub-group analysis revealed that only C4.4A-positive/TNC-positive cases had a significantly worse prognosis when compared to C4.4A-negative/TNC-negative cases (Fig. 6, P=0.005).

Discussion

Esophageal cancer is the eighth most common cancer worldwide and the sixth most common cause of death from cancer (26). In Asian countries esophageal squamous cell carcinoma (ESCC) is more prevalent than adenocarcinoma and accounts for >90% of esophageal carcinomas. ESCC is usually diagnosed at an advanced stage, which leads to a 5-year survival of only 10% (27). To improve the unfavorable outcome of ESCC, it is essential to explore the molecular basis of the underlying mechanism of this disease.

HCT116 colon cancer cells represent a positive control for the C4.4A protein (7,8). We previously observed that the C4.4A protein was located in the cytoplasm of HCT116 cells on collagen gels, but it was translocated onto the plasma membrane when HCT116 cells were cultured in the collagen matrix (7). We also found that C4.4A mRNA levels increased 2.6-fold in 3D collagen cultures (data not shown). Based on these findings, we hypothesized that membranous C4.4A might exert certain roles when cells are grown in the 3D collagen matrix. To examine this possibility, we performed a PCR array analysis using HCT116 cell cultures grown for 48 h in 3D-collagen matrix after C4.4A-siRNA treatment. TNC was one of the notable genes that increased in the 3D condition compared to the 2D culture, but was reduced when C4.4A was knocked down in the 3D condition.

TNC is an extracellular matrix protein composed of six monomers linked at their N-termini with disulfide bounds to form a 1080-1500-kDa hexamer and various solid tumors express high level of TNC (12-19,28,29). TNC is secreted from both tumor cells and myofibroblasts. The most prominent effects of TNC are anti-adhesion and inhabitation of cell attachment, both of which favor cancer cell motility and invasion (30,31). Furthermore, TNC promotes malignant transformation, uncontrolled proliferation, metastasis, angiogenesis, drug resistance and escape from tumor immunosurveillance (15,32,33). Knockdown experiments for TNC in glioblastomas and breast cancer cells caused inhibition of tumor growth, migration, invasion and metastasis (12,18,34).

Recently, we have shown that C4.4A expression is associated with a poor prognosis of ESCC (10), but the underlying mechanism of how C4.4A influences the prognosis of ESCC remains unknown. Based on the *in vitro* observation that TNC levels increased in parallel with lentivirus-mediated introduction of the C4.4A gene in TE8 esophageal cancer cells, we examined TNC and C4.4A expression in 111 ESCCs. As results, we found that TNC expression was significantly associated with C4.4A expression in clinical ESCC samples (Fig. 5, P=0.007), suggesting that there may be a functional role for the C4.4A to induce TNC *in vivo*. Among the C4.4A positive ESCCs, only double positive C4.4A and TNC group had poorer prognosis (Fig. 6).

In conclusion, we revealed for the first time that the TNC-positive group (24.3%) had significantly poorer prognosis than the TNC-negative group in 5-year OS. It is also postulated that TNC may partly account for the C4.4A-related unfavorable outcome in ESCC patients.

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