RESEARCH COMMUNICATION

Expression Pattern of Tumor Endothelial Marker 8 Protein in Gallbladder Carcinomas

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Abstract

Tumor endothelial marker 8 protein (TEM8) is highly specific to tumor angiogenesis and is not required for normal adult angiogenesis and hence might prove to be a target for anti-angiogenic therapies in the future. We here evaluated protein and gene expression patterns in human endothelial cells of benign gallbladder - gallstone diseases (GSDs) and gallbladder carcinomas (GBCs) using immunostaining, immunofluorescence and western blotting techniques. Subjects comprised 175 GBC patients, 38 males and 137 females, aged 30–85 years (mean age 50.3±13.4 years) and twenty with GSDs, aged 30-75 years, (51.4±10.0 years) for comparison (male 4/20 and females 16/20). TEM8 protein expression increased significantly (p<0.0001) with increasing stage of GBC and was mostly limited to endothelial cells, although there was no significant change with the grade. Interestingly, only 80-85 kDa and 60 kDa isoforms of TEM8 increased significantly whereas 45 kDa isoform was absent in GBCs. Conclusions- These results suggest that TEM8 plays an unknown important biological role to promote tumor angiogenesis in GBC.

Keywords: Gallbladder carcinoma - angiogenesis - endothelial marker - new vasculature

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Introduction

Gallbladder cancer (GBC) is the third most common form of digestive tract malignancy. It is singularly characterized by diagnosis in late stage leading to unsatisfactory treatment, poor prognosis and palliative management strategies. GBC is a relatively uncommon neoplasm in the world, but there is a considerable geographic variation in its incidence (Shukla, 1981; 2006). It is common in Japan, India and Chile (Lazcano-Ponce, 2001). It is the most common biliary tract malignancy and the fifth most frequent gastrointestinal malignancy (Darabos, 2004). In the United States, around 7500 new cases of biliary tract cancer are diagnosed per year, and 5000 of those cases are diagnosed as gallbladder cancer (Kerbel, 2002). GBC is a highly aggressive malignancy that is associated with approximately 2500 deaths per year (Kerbel, 2002). GBC mortality remains high due to its aggressive and silent nature.

Angiogenesis is defined as the development of new blood vessels from pre-existing vessels and is characterized by expression of the endothelium by propagation migration and remodeling and is a key to cancer development and mainly metastasis. The regulatory mechanism at molecular and cellular level of angiogenesis is complex with a rising list of possible regulators (Maurya, 2009; 2010). It is known that to conquer tissue death by hypoxia, tumor growth beyond 1-2 mm³ is dependant upon the development of fresh vasculature. Thus the inhibition of tumor angiogenesis as an attractive anticancer strategy has gained widespread support from cancer researchers and clinicians (Thijssen, 2006).

Serial analysis of gene expression (SAGE) on human normal colonic mucosa or colorectal tumor endothelial cells identified a new class of tumor endothelial markers (TEM1-TEM9) (St Croix, 2000). It is predicted that TEM8 function and expression has been associated with development of the vascular system and with tumor angiogenesis and exists in different forms at the cell surface, a structure dependent on interactions with components of the actin cytoskeleton (Yang, 2010). TEM8 is selectively upregulated in endothelial cells during blood vessel formation (St Croix, 2000; Carson-Walter, 2001). TEM8 is especially interesting because of its cell-surface localization and high amino acid sequence conservation between human and mouse (Carson-Walter, 2001). TEM8 appears to be the only tumor endothelial marker characterized to date that is not expressed in either the corpus luteum or healing wounds, suggesting that it is highly specific to tumor angiogenesis and not required for normal adult angiogenesis (St Croix, 2000; Carson-Walter, 2001).

Targeted disruption of tumor vasculature is an area of growing interest in cancer biology and therapeutics
Sanjeev Kumar Maurya et al

(Duan, 2007; Venanzi, 2010). Taken together, all of these characteristics make TEM8 particularly useful in the development of neovascularization-targeted antitumor agents. In the present study, we assessed TEM8 protein expression in GBC (cases) and benign Gallstone Disease (GSD) (controls). Currently, there are three reported transcript variants of TEM8 and the protein products of these variants are 80 kd, 60kd and 45kd respectively. Thus, it is not yet clear that which of these three known variants plays a crucial role in angiogenesis and tumor progression. Although the microvessel count in some kinds of solid tumor was shown to correlate with clinical outcome, little is known about its significance in gallbladder carcinoma.

To answer these questions in the present work we conducted immunohistochemistry, immunoflotrescence and western blotting from tumor cell lysate of GBC tissue and compared it with normal GSD tissue lysate as the control.

Materials and Methods

Patients and sample collection

The subjects consisted of a total of 175 GBC patients (cases) 38males and 137 females, aged 30-85 years (mean age 50.25±13.44 years). Twenty patients with GSD, aged 30-75 years, (mean age 51.37±10.04 years) were taken as controls (male 4/20 and females 16/20). This study was carried out between December 2006 and January 2008 in the Department of Surgical Oncology, Institute of Medical Sciences, Banaras Hindu University, Varanasi (India). The patients were not treated by surgery, chemotherapy or radiotherapy prior to their enrolment in this study. The diagnosis was confirmed by histopathology of excised specimen of gallbladder or biopsy in all patients. Histopathology was done in Department of Pathology, Institute of Medical Sciences, Banaras Hindu University, Varanasi (India). Immunohistochemistry (IHC), Immunoflotrescence (IF) and western blotting was carried out with the help of Department Zoology, Banaras Hindu University, Varanasi (India). All the patients were examined by clinical history, physical examination and routine blood test and radiological imaging of gallbladder. A pre-tested proforma was made to record all the data. The study was approved by the human ethical committee of our Institute.

Immunohistochemistry

All specimens were fixed in 10% formalin, embedded in paraffin, serially sectioned into 4 mm thick cuts and mounted on polysine coated slides. The slides were immersed for 20 min in 0.3% hydrogen peroxide in methanol to deplete the endogenous peroxidase. After washing, they were incubated with a protein-blocking agent (1% normal rabbit serum diluted in PBS) for 5 min. Primary antibodies against TEM8 core protein (monoclonal antibody, rabbit anti-human TEM8 (kindly gifted by Prof Brad St. Croix, PhD, Tumor Angiogenesis Section, National Cancer Institute at Frederick, USA)) were used at a dilution of 1:100. For the negative controls, the primary antibody was replaced with PBS. The slides were incubated with primary antibody in a humid chamber for 1 h, washed with PBS for 15 min, and underwent changing of the buffer three times. Then, biotinylated secondary antibodies (Santa Cruz Biotechnology Inc, USA) were applied (1:500 dilution) for 10 min at room temperature. After washing, streptavidin peroxidase reagent was applied and the samples were incubated for 10 min at room temperature. Lastly, the slides were visualized by incubation within solution containing 0.3% hydrogen peroxide and diaminobenzidine tetrahydrochloride in PBS. Counterstaining was performed with haematoxylin, prior to mounting and observed in Light microscope for image analysis and quantification. Immunostaining intensity of TEM8 in gallbladder tissue was graded as strong (+++), moderate (++), weak (+) or nil (0) staining.

Immunoflotrescence

For immunoflotrescence staining sections were deparaffinsed, rehydrated, endogenous peroxidase blocking and antigen retrieval according to method describe in immunohistochemistry section. Non specific blocking was done by blocking buffer (1% normal rabbit serum and 0.5% Tween-20 in PBS) for overnight at 40C. Anti-TEM8 primary antibody grown in rabbit was diluted (1:1000) in blocking buffer and was incubated with section for 1 hour at room temperature. After three wash with PBS+ 0.5% Tween-20, anti-rabbit FITC-conjugated secondary antibody (1:800) were diluted in blocking buffer was applied on sections and incubated for 30 minutes at room temperature in a humid chamber. The sections were washed three times with PBS and 0.5% Tween-20. Slides were mounted by 50% glycerol and covered by coverslip and seal edges with clear nail polish. Images were taken by fluorescence microscope for documentation and image analysis. Staining was analyzed using an automated imaging system with an Olympus IX50 microscope and Olympus imaging-analysis software (Olympus, Hamburg,Germany).

Western blotting

Tissue were grinded in liquid nitrogen and homogenized in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1% (w/v) Triton X- 100) followed by centrifugation at 5000g for 10 min at 40C. Clear supernatant were taken for protein estimation and SDS PAGE. Total 50 µg protein were resolved using 3% stacking gel and 10% resolving gel. Proteins were electrophoretically transferred to Hybond-C Super membranes (Amersham Pharmacia Biotech Ltd., UK), and blocked in 5% (w/v) fat free dried skimmed milk in phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO3, 1.4 mM KH2PO3, pH 7.3) for 1 h. Membrane was incubated with primary anti-TEM8 antibody (1:2000 dilutions) for two hours at room temperature followed by washing three times with PBS. β-Tubulin antibody (1:5000) was used as a control to confirm equal gel loading of the samples. Immunoblots were washed and incubated with appropriate secondary Ab (1:10,000) and visualized using SuperSignal West Pico chemiluminescence reagents (Pierce, Rockford, IL) and image was captured on X-ray film. Blot densitometry was
Expression Pattern of Tumor Endothelial Marker 8 Protein in Gallbladder Carcinomas

Statistical analyses

The degree of immunohistochemical and immunofluorescence staining for TEM8 proteins in GBC in relation to different stage, grade and GSD was assessed with the chi square test. The densitometric data obtained from western blot were analyzed in different stages and grades of GBC and in GSD were subjected to parametric statistical analysis by two tailed unequal variance student ‘t’ test was used to establish statistical significance using SPSS 11.00 software (USA). Differences were considered significant when p<0.05.

Results

To evaluate TEM8 protein expression level, we assessed and compared its expression in different stages and grades of GBC (Figure 1). Another endothelial marker CD31 was taken as positive control as vascular density marker. TEM8 immunostaining in GBC tissue showed upregulated endothelial staining. Average TEM8 expression clearly increased with GBC stage, and it was observed that variation in TEM8 expression also increased at the same time (Figure 2). In GBC, TEM8 was expressed in 10.4% (3/29) of cases in T2 tumor, 79.5% (62/78) in T3 tumor and 84.8% (62/66) in T4 tumors. There were only two T1 cases and in both TEM8 expression was absent. In T2 tumor, two cases showed weak expression and one showed moderate expression. In T3 tumors, expression was weak in 32.05 % (25/78), moderate in 32.05% (25/78) and strong in 15.3% (12/78) of cases. In T4 tumors, 28.8% (19/66) cases showed weak, 36.4% (24/66) moderate and 19.7% (9/66) strong expression of TEM8 (Figure 3).

With respect to histological grade of GBC, TEM8 positivity was observed in 63.3% (38/60) well differentiated, 67.2% (41/61) in moderately differentiated, and 70.3% (38/54) in poorly differentiated GBC (Figure 4).

To verify the result of Immunostaining and localization of TEM8 protein distribution in GBC samples, immunofluorescence was done with FITC conjugated secondary antibody. For this we selected 10 samples each of T2, T3, T4 GBC tissue and GSD for analysis. We found that TEM8 staining increased from T2 to T4 tumor. TEM8 was localized to endothelial cells and stained whole endothelial cells of GBC tissue (Figure 5).

The role of different TEM8 isoforms in normal physiology or in cancer remains to be determined. To know the status of these three isoforms in gallbladder is also important. Western blotting was performed with 50μg protein of whole cell lysate from 66 T4 cases of GBC and 25 GSD tissues. β-actin was used as a loading control. Western blot showed the presence of two (80-85 and 60 KDa) isoforms and absence of 45 KDa isoform in GBC cellular lysate. In GSD tissue, expression of TEM8 isoforms was undetectable. Significant increase in TEM8 expression (p<0.0001) was observed in T4 as compared to GSD tissue. Figure 6 shows that TEM8 expression was higher in well differentiated and moderately differentiated}

Figure 1. Immunohistochemical Localization of CD31 and TEM8 Protein in GBC (10X magnification)

Figure 2. Box-Plot of TEM8 Expression in GBC from T1 to T4 Stages

Figure 3. Immunohistochemical Expression of TEM8 in Different Tumor Stages. On the basis of the intensity of TEM8 expression, each stage is categorized into weak, moderate and strong. Results are percentages. *Significantly different from T2 tumor p<0.0001

Figure 4. Immunohistochemical Expression of TEM8 in Different Tumor Grades.

drives TEM8 overexpression. The mechanism behind this overexpression and stage dependence is still unknown. Further, we did not find any significant differences of TEM8 staining between different tumor grades. This shows that TEM8 expression is independent of tumor differentiation.

TEM8 expression was at first documented as exclusive to angiogenic vessels in vivo (St Croix, 2000; Davies, 2004), yet no functional significance has been assigned to TEM8 as a regulator of endothelial cell biology. TEM8 may involve supporting endothelial cell migration through enhancing cell–matrix interactions on collagen (Nanda, 2004). TEM8 may play a fundamental role in angiogenesis, converting the normal quiescent endothelial cell state to one that is reactive and highly active cells. Presented data support previous findings showing TEM8 expression is precise to the tumor vasculature and indicates TEM8 expression is likely to be a necessary factor of the angiogenic response (Mooney, 1995).

To support our immunohistochemistry results we did immunofluorescence to get more precise localization of TEM8. Our Immunoflorescence results confirmed the immunohistochemistry findings that TEM8 protein expression is limited to tumor endothelial cells only. Quantitative measurement of TEM8 fluorescence intensity was also done in endothelial cells and epithelial cells. The quantification tests revealed that around 8 fold (p<0.002) more intense staining was present in the tumor endothelial cells compared to the normal endothelial cells of the control. The absence of TEM8 expression in T1 stage was surprising, but not unexpected despite earlier observations indicating that the TEM8 is not expressed in dormant endothelial cells in vivo (Carson-Walter, 2001; Nanda, 2004). Similarities in the phenotypes of angiogenic and embryonic endothelial cells have been described and TEM8 expression has been previously detected on endothelial cells in embryonic mouse liver by in situ hybridization (St Croix, 2000). The ability of extracellular matrix molecules or cleaved extracellular matrix fragments to regulate angiogenesis is well established (Grant, 1997; Marneros, 2001; Kalluri, 2003). Thus, it is possible that the interaction between TEM8 and collagen α3(VI) is important for angiogenesis. Hotchkiss (2005) demonstrated that TEM8 stimulates endothelial cell adhesion and migration by cell matrix interactions on collagens. Identification of natural ligands for TEM8 could provide important clues about its function. Nanda (2006) also demonstrated that Collagen α3 (VI) is a natural interacting partner of extracellular domain of TEM8 protein. It is known that collagen α3(VI) is up-regulated in healing wounds, which are known to be rich in neovascularature. TEM8 functions as an autonomous adhesion molecule coupling binding of an immobilized extracellular ligand and cell spreading through association to the actin cytoskeleton. This evidence suggests interaction with molecules of the extracellular matrix (ECM); however the implications of these interactions for cell adhesion remain undetermined. TEM8 also increases endothelial cell chemotactic activity on collagen substrata; however the mechanism for this function remains unclear (Werner, 2006). Our result conform the Rmali (2004)

**Discussion**

Out of other tumor endothelial markers, TEM8 is particularly an attractive candidate for targeted therapy because of its potential for minimal cross reactivity with other normal tissues (Bradley, 2001; Scobie, 2003). For this, demonstration of TEM8 expression in GBC is crucial against non cancerous gallbladder (GSD). The pattern of TEM8 expression in GBC and GSD in the present study suggests that it is expression is found to occur only in the tumor endothelial cells and not in the normal endothelial cells. The interesting observation of our study was TEM8 protein expression pattern. Its intensity significantly increases with tumor stage. This indicates that tumor stage
finding in colon cancer, in which they demonstrated, that the level of tumour endothelial marker 8 (TEM-8) in colorectal cancer tissues was found to be elevated, and also significantly raised in cancer patients with both nodal involvement, and/or in those who had poor prognosis (Venanzi, 2010).

Cytoplasmic amino acid sequence and In silico study reveals that TEM8 has a consensus signal for basolateral sorting and for phosphorylation. The cytosolic domain is palmitoylated and ubiquitinated, modifications controlling endocytosis of the receptor (Abrami, 2006). However, no other known protein motifs or interacting proteins known till date, so far that could give additional clues about the function of the receptor cytosolic domain. To understand about role of cytosolic domain of TEM8 receptors during tumor angiogenesis it is necessary to know the expression pattern of protein variants generated by differential splicing (Scobie, 2003) during transduction. Importantly, whereas this domain is not required for toxin delivery (Liu, 2003), it could potentially play a determinant role in regulating receptor localization or contribute to the host response triggered by toxin internalization. MIDAS mutant of ANTXR1 (T118A) was found to retain normal metal ion binding and secondary structure but failed to bind PA, consistent with a locked inactive state. So it is important to regulate toxin binding for the design of toxin inhibitors and for the targeting of ANTXR1 for antitumor therapies (Ramey, 2010).

Further, to know the expression pattern of these three known protein isoforms of TEM8 we performed western blotting with monoclonal anti-TEM8 antibody. To our knowledge this is the first study to show the expression of TEM-8 at the isoform level in gallbladder cancer and normal background tissues (GSD). Out of three isoforms we observed only 80 kDa and 60kDa isoform expressed in GBC where as we did not find any TEM8 isoform expression in GSD tissue lysate. This experiment gives two important conclusions first, our above immunohistochemistry in the present study. This research work was done with the financial help of University Grant Commission, India vide Junior and Senior Research Fellowship.

References

Sanjeev Kumar Maurya et al


