

Expression Patterns of Thymosin β 4 and Cancer Stem Cell Marker CD133 in Ovarian Cancers

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Abstract Thymosin β 4 (T β 4), a small acidic actin binding peptide, is overexpressed in a side population of cancer stem cells and CD133-positive colorectal cancer stem cells. In order to understand the relationship between T β 4 and CD133, we studied the expression patterns of T β 4 and CD133 in ovarian cancers. The expression patterns of T β 4 and CD133 were studied in normal ovaries, primary ovarian cancers, metastatic ovarian cancers, primary stomach cancers, and normal stomachs by Western blot and immunohistochemistry. Expression patterns and co-localization of T β 4 and CD133 were examined by immunofluorescence and confocal laser-scanning microscopy. T β 4 is overexpressed in primary ovarian cancers, but not in primary stomach

cancers, when compared with normal controls. However, T β 4 levels in metastatic stomach cancers to the ovary are significantly upregulated compared with levels in normal stomachs and primary stomach cancers. These results suggest that T β 4 levels are related to tumorigenesis in ovarian cancers and metastasis in stomach cancers. The expression of T β 4 in normal ovaries and normal stomachs was weak, but was co-localized with CD133 expression. T β 4 expression was also co-localized with CD133 expression in primary ovarian carcinomas, metastatic ovarian cancers from stomach cancers and primary stomach cancers. These data suggest that T β 4 expression is strongly related to CD133 expression and is a characteristic of stem cells or cancer stem cells.

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Introduction

Thymosin β 4 (T β 4), a small acidic peptide first isolated from calf thymus, has been postulated to be a thymic maturation factor [1]. However, T β 4, along with other members of this peptide family, were later identified as intracellular G-actin sequestering molecules presented in almost every type of cell [2]. T β 4 prevents the formation of actin microfilaments by complexing with monomeric G-actin in a 1 : 1 ratio [3]. On the other hand, T β 4 also supplies a pool of actin monomers for polymerization when the cell needs filaments [4] and stimulates cell migration [5]. Many reports have shown that exogenous T β 4 stimulates cell migration and plays physiological roles in various cellular processes, including angiogenesis [5–7], wound healing [8, 9], and hair growth [9–11]. Other studies have shown that T β 4 also plays a role in anti-apoptosis [12–15] and anti-inflammation activities [16, 17].

Increased T β 4 expression has been reported in breast, ovarian and uterine cancers [18, 19], and is associated with metastasis in human colorectal, renal and lung cancer [20–22]. Experimentally, T β 4 expression has been shown to enhance tumor growth and metastasis in mouse fibrosarcoma and melanoma cells [23, 24], and in vivo tumor growth and invasion of human colon carcinoma cells [25]. Conversely, antisense T β 4 expression induced fibrosarcoma tumor formation and metastasis [23]. Together, these results demonstrate the importance of intracellular T β 4 overexpression in cancer.

Cancer is increasingly being viewed as a stem cell disease [26]. According to the cancer stem cell (CSC) hypothesis, cancer is initiated and maintained by a small population of tumor-initiating cells endowed with the ability to self-renew and differentiate in nontumorigenic cells. The CSC model has been supported by studies showing that the growth of several types of tumors depends on a small subset of stem-like cancer cells displaying many features in common with their nontransformed counterparts [27]. Recent reports have demonstrated that a small number of undifferentiated cells within colon cancer are able to proliferate indefinitely and reproduce the tumor in immunocompromised mice, while generating progeny of more differentiated cells devoid of tumorigenic potential [26, 28, 29]. The identification of colorectal CSCs (CR-CSCs) provides strong support for the hierarchical organization of human colon cancer, implying the necessity to define the mechanisms responsible for unrestrained proliferation and high malignancy of CR-CSCs. Studies were conducted to identify the CRC markers and these studies demonstrated that

CRCs are located in a side population that possess CD-133 as a cancer stem cell marker [27, 30, 31]. CD133 (also known as Prominin-1 or AC133), a transmembrane pentaspan protein, was initially described as a surface antigen specific for human hematopoietic stem cells [32, 33], and is a cancer stem cell marker identified in a putative cancer stem cell population from malignant tumors of the brain [34, 35], prostate [36], liver [37, 38], pancreas [39], lung [40], and colon [28, 41, 42]. In a recent study, mass spectrometry-based stable isotope labeling by amino acids in cell culture was used to identify differentially expressed proteins in side population cells from two breast cancer cell lines MCF7 and MDA-MB231, and T β 4 expression was elevated in a small cell population [43]. Moreover, a study investigating the role of T β 4 in promoting tumorigenesis of colorectal cancer stem cells (CR-CSCs) showed that CR-CSCs sorted by CD133 from different patients had higher T β 4 levels than normal epithelial cells. Further, a lentiviral strategy was used to downregulate T β 4 expression in CR-CSCs, resulting in a lower capacity to grow and migrate in culture and, interestingly, a reduced tumor size and aggressiveness of CR-CSC-based xenografts in mice [44].

Most (more than 90 %) ovarian cancers are classified as “epithelial” and are believed to arise from the surface (epithelium) of the ovary [45]. Ovarian cancer can also be a secondary cancer, resulting from metastasis from a primary cancer elsewhere in the body. Seven percent of ovarian cancers are due to metastases, while the rest are primary cancers. Common primary cancers that metastasize to the ovary are breast cancer and gastrointestinal cancer. Surface epithelial-stromal tumors can originate in the peritoneum (the lining of the abdominal cavity), in which case the ovarian cancer is secondary to primary peritoneal cancer [46].

In the present study, we first investigated the expression patterns of T β 4 and CD133 in normal ovaries, primary ovarian cancers, ovarian cancers metastasized from stomach cancers, primary stomach cancers and normal stomachs by Western blot and immunohistochemical analysis. We also defined the relationships of T β 4 and CD133 expression in normal ovaries, primary ovarian cancers, ovarian cancers metastasized from stomach cancers, primary stomach cancers and normal stomachs by analyzing co-localization of T β 4 and CD133 expression with immunofluorescence analysis.

Materials and Methods

Patient Sample

We analyzed the expression pattern of T β 4 and CD133 by Western blot and immunohistochemistry in fresh tissues

from 22 patients and slides from paraffin blocks of 65 patients. The details of the patient sample are summarized in Table 1.

Western Blot Analysis

Western blot analysis was conducted as previously described [24]. Briefly, 50 μ g of tissue extract were prepared using PRO-PREPTM Protein Extraction Solution (Intron Biotechnology, Kyunggi, Korea) and separated by electrophoresis on a Novex 4–12 % Bis–Tris gel (Invitrogen, Carlsbad, CA, USA). The protein concentrations were determined using the bicinchoninic acid protein assay system (Pierce, Rockford, IL, USA), and equal amounts of each sample were separated by electrophoresis on Novex 4–12 % Bis–Tris gels. Equal protein loading was confirmed by Coomassie blue staining of duplicate gels after electrophoresis. For T β 4, the gels were incubated for 1 h in phosphate-buffered saline (PBS) containing 10 % glutaraldehyde (Sigma–Aldrich, St. Louis, MO, USA) and then washed three times for 20 min each in PBS. The gels were incubated in blotting buffer containing 1 $\times$ NuPAGE® Bis–Tris transfer buffer (Invitrogen, Carlsbad, CA, USA) and 20 % methanol for 30 min at room temperature. Proteins were transferred to a nitrocellulose membrane (Invitrogen, Carlsbad, CA, USA) by electrotransfer. For detection of T β 4, small proteins were fixed on the membrane by UV cross-linking.

The membrane was pre-incubated for 2 h in Tris-buffered saline (TBS) containing 5 % skim milk and 0.05 % Tween 20 (TBS-T). The membrane was incubated overnight at 4°C in TBS-T plus anti-T β 4 (mouse monoclonal anti-T β 4, 1:1,000 dilution; Abcam, Cambridge, MA, USA), and anti-CD133 (rabbit polyclonal anti-CD133 antibody, 1:1,000 dilution; Abcam, Cambridge, MA, USA). The membranes were washed five times with PBS-T and then incubated with a

species-appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 1 h at room temperature. The membranes were washed five times with TBS-T, and bound antibody was detected with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Western blot analysis was performed more than three times using samples from three different animals, and the band densities were quantitated by image analysis software (Image J; NIH, Bethesda, MD, USA). The specificities of all antibodies were confirmed by positive controls.

Immunohistochemistry

For immunohistochemistry, tissue slides were deparaffinized and hydrated. For antigen retrieval, slides were immersed in citrate buffer (0.01 M, pH 6.0) and heated twice in a microwave (700 W or higher) for 5 min. Then, the slides were quenched with endogenous peroxidase by incubation with 3 % hydrogen peroxide solution for 5 min and washed three times in PBS for 5 min. Slides were immunostained with rabbit polyclonal antibody to T β 4 (1:1,000 dilution; ALPCO Diagnostics, Windham, NH, USA) or CD133 (1:100 dilution, Abcam Inc., Cambridge, MA, USA) at 4 °C overnight. After primary antibody incubation, slides were washed three times in PBS for 5 min and incubated with secondary antibody (DAKO REAL EnVision, HRP rabbit/mouse, CA, USA) for 1 h. After secondary antibody incubation, slides were washed four times in PBS for 5 min each and the color reaction was developed with Dako's EnVision™ G|2 Doublestain System (DAKO, Carpinteria, CA, USA). T β 4 was stained with DAB (diaminobenzidine) (DAKO, Carpinteria, CA, USA) and VEGF was stained with permanent red (DAKO, Carpinteria, CA, USA). Slides were counterstained with Meyer's hematoxylin (DAKO,

Table 1 Patient population for experiments

Experiments	Type of tissue	Tissue condition	Number of patients
Western blot	Normal ovary	Fresh	4
	Primary ovarian cancer	Fresh	5
	Metastatic ovarian cancer	Fresh	5
	from stomach cancer		
	Normal stomach	Fresh	4
	Primary stomach cancer	Fresh	4
Immunohistochemistry	Normal ovary	Paraffin	4
	Primary ovarian cancer	Paraffin	19
	Metastatic ovarian cancer	Paraffin	18
	from stomach cancer		
	Normal stomach	Paraffin	12
	Primary stomach cancer	Paraffin	12

Carpinteria, CA, USA) for 10 s, dehydrated, and mounted with Permount (Fisher Scientific, Pittsburgh, PA, USA).

Immunofluorescence

Paraffin-embedded sections were deparaffinized and hydrated. For antigen retrieval, slides were immersed in citrate buffer (0.01 M, pH 6.0) and heated twice in a microwave (700 W or high) for 5 min each. Immunofluorescence was analyzed as described previously [47]. In short, slides were permeabilized by incubation in PBS containing 0.1 % Triton X-100 for 5 min and incubated with 10 % normal serum in PBS for 1 h to block nonspecific antibody binding. Slides were then incubated with a mixture of mouse anti-T β 4 monoclonal (1:100 dilution; Abcam Inc., Cambridge, MA, USA) and rabbit anti-CD133 polyclonal antibody (1:100 dilution; Abcam Inc., Cambridge, MA, USA) at 4°C overnight. After primary antibody incubation, slides were washed three times in PBS for 5 min each and incubated with secondary antibodies (Alexa Fluor 546 anti-mouse antibody and Alexa Fluor 488 anti-rabbit antibodies; Invitrogen, Carlsbad, CA, USA) for 1 h. The slides were observed for epifluorescence with a confocal laser-scanning microscope.

Semiquantitative Assessment

For protein expression assessment for immunohistochemistry, staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). Focal intensity of expression was scored and the score was added to the general score (0.5, focally weak; 1.5, focally moderate; 2.5, focally strong). Three well-trained and blinded observers read the slides and scored the expression of T β 4 and CD133.

Statistical Analysis

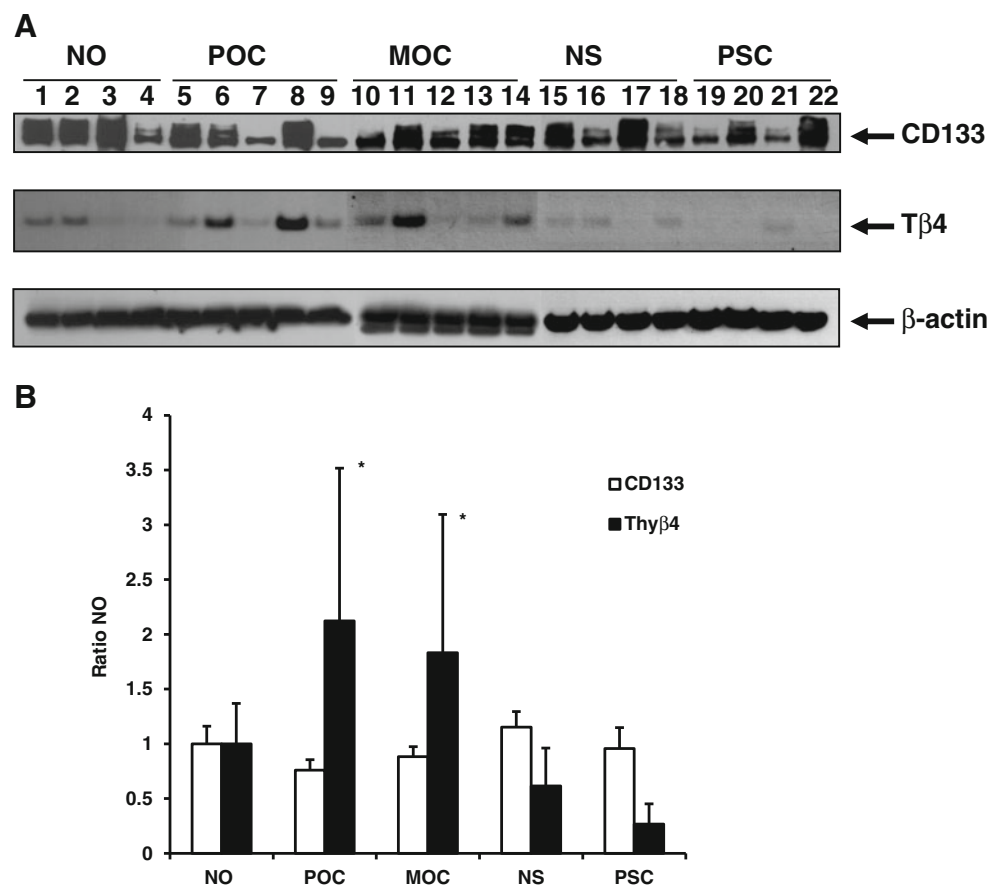
The statistical significance of differences among groups was determined using a two-tailed Student's *t*-test. *P* values less than 0.05 were considered statistically significant.

Results

Expressions of T β 4 and CD133 in Ovarian Cancers

In order to identify the expressions of T β 4 and CD133 in ovarian cancers, T β 4 levels were checked in normal ovaries

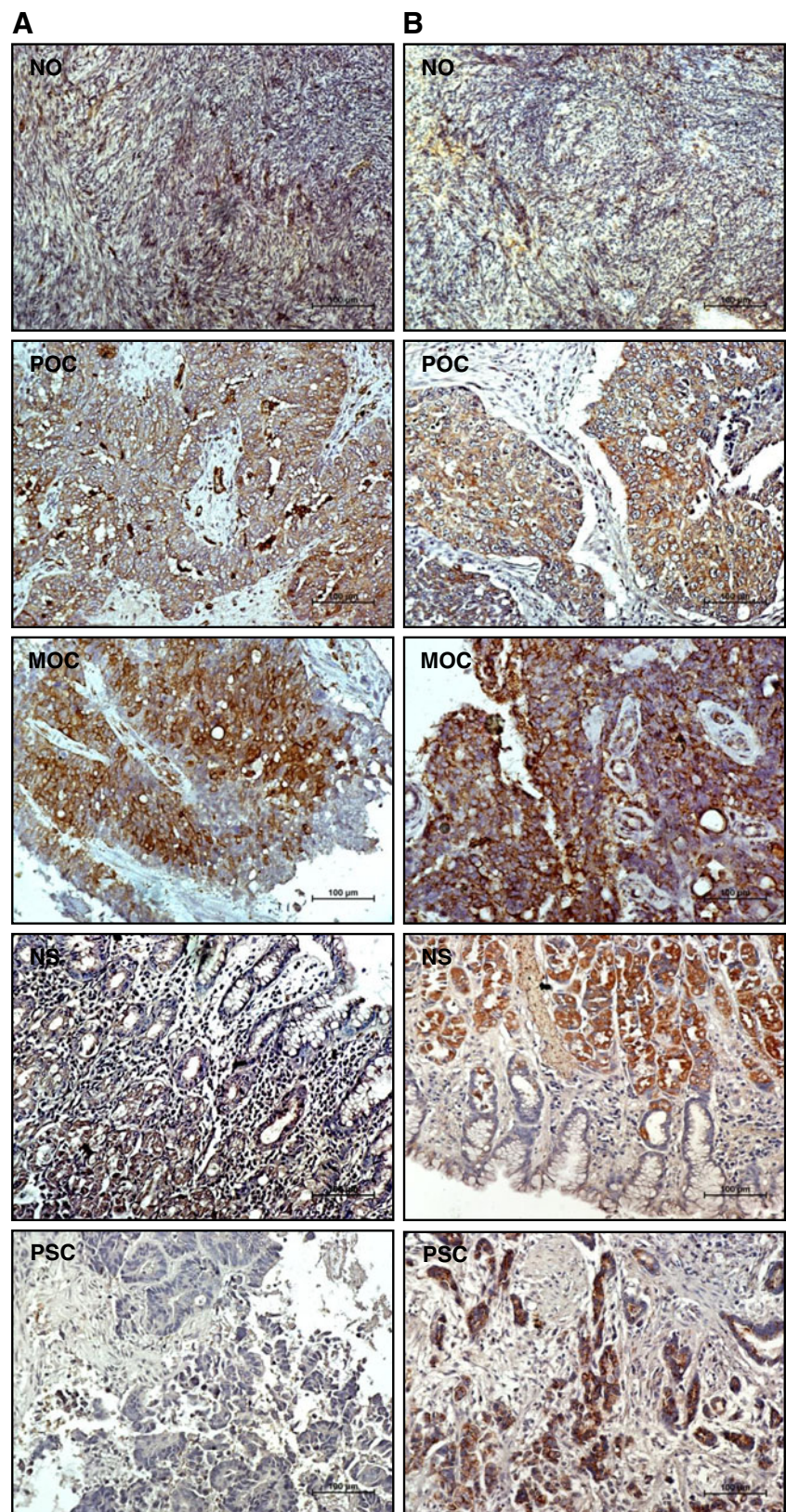
Fig. 1 Expression of T β 4 and CD133 in ovarian cancers. Expressions of normal ovaries (NO), primary ovarian cancers (POC), and metastatic ovarian cancers (MOC) were analyzed by western blotting (**a**), and relative bands densities were compared (**b**). Expression levels in normal stomachs (NS) and primary stomach cancers (PSC) were also compared as a control for metastatic ovarian cancers. Lanes 1 to 22 indicate the individual patients. Western blot was performed in more than three independent experiments and the band densities were quantitated by image analysis software (Image J, NIH, Bethesda, MD, USA). Band densities of western blot products were normalized to β -actin and presented relative to NO



(NO), primary ovarian cancers (POC), and metastatic ovarian cancers from stomach cancers (MOC) by Western blot.

The expressions of T β 4 and CD133 in normal stomachs (NS) and primary stomach cancers (PSC) were also checked

Fig. 2 Expression patterns of T β 4 (**a**) and CD133 (**b**) in ovarian cancers. Expression in normal ovaries (NO), primary ovarian cancers (POC), and metastatic ovarian cancers (MOC) was analyzed by immunohistochemical staining. Expression levels in normal stomachs (NS) and primary stomach cancers (PSC) were also compared as a control for metastatic ovarian cancers. Original magnification X 200. Comparison of the T β 4 (**c**) and CD133 expression (**d**) in ovarian cancers. Immunohistochemical staining in NO, POC, MOC, NS, and PSC samples were analyzed. For protein expression assessment, the staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). Focal intensity of expression was scored as one-half and added to the total score. Three investigators read the slides and scored the expression of T β 4 and CD133. The mean expression scores were calculated and compared



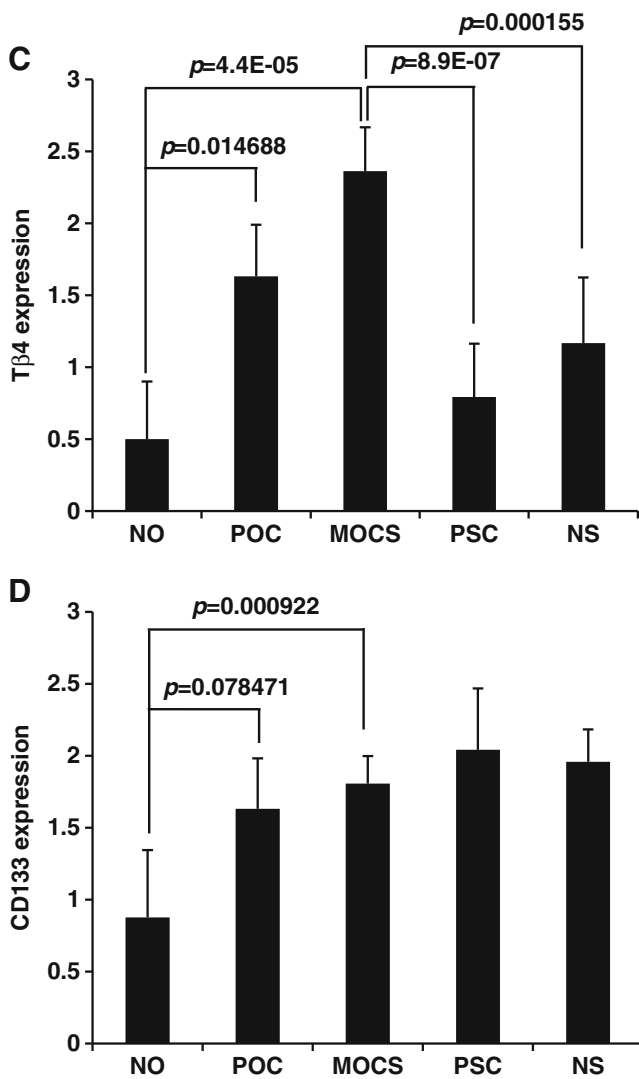


Fig. 2 (continued)

as controls. As shown in Fig. 1a, the expression levels of Tβ4 were different for each individual, but were up-regulated in POC and MOC in comparison to levels in NO, NS and PSC. CD133 expression pattern also differed according to each individual increase in PSC, in comparison with NO, but did not change significantly in MO, in comparison with NS and PSC (Fig. 1b). These results indicate that the expression of Tβ4 is elevated in primary ovarian cancers, but does not change in primary stomach cancers, in comparisons with normal controls. However, Tβ4 levels in metastatic ovarian cancers from stomach cancers are significantly up-regulated compared with normal stomach and primary stomach cancers. These results suggest that Tβ4 levels are related to tumorigenesis in ovarian cancers and metastasis in stomach cancers.

We conducted immunohistochemistry with four NO, 19 POC, 18 MOC, 12 NS, and 12 PSC samples to

analyze the expression pattern and location of Tβ4 and CD133. All mean scores of expression were calculated and compared, as shown in the graphs of Fig. 2c and d. The expression of Tβ4 was significantly up-regulated in POC ($p=0.015$), and Tβ4 expression was greatly increased in MOC compared with NO ($p=4.4E-05$), PSC ($p=8.9E-07$) and NS ($p=0.000155$), respectively (Fig. 3). The expression level of CD133 was up-regulated significantly in POC and MOC compared with NO. However, we did not observe differences in expression levels of CD133 when comparing MOC, PSC and NS (Fig. 2c and d). These results agree with those of western blot analyses. An example of Tβ4 expression in each tissue is shown in Fig. 2a. Tβ4 was weakly expressed in NO, but highly expressed in POC and MOC. The Tβ4 expression level in normal stomach was weak and limited to the deep glands. PSC also showed weak expression of Tβ4. CD133 levels in each tissue are shown in Fig. 2b. CD133 was weakly expressed in NO, but highly expressed in POC and MOC. NS and PSC showed strong CD133 expression in the deep glands.

Localization of Tβ4 and CD133 in Ovarian Cancers

Immunofluorescence was conducted with dual primary antibodies of anti-Tβ4 and anti-CD133 to analyze the co-localization of Tβ4 and CD133 in each tissue. As shown in Fig. 3, Tβ4 and CD133 expression was co-localized with both normal and cancer tissues. Tβ4 expression in NO and NS was weak, but co-localized with CD133 expression. Tβ4 also co-localized with CD133 in POC, MOC and PSC. These data suggest that Tβ4 expression is strongly related to CD133 expression and is a characteristic of stem or cancer stem cells.

Discussion

Cancer has long been viewed as an exclusively genetic disorder. The model of carcinogenesis describes tumor formation through the sequential accumulation of mutations in oncogenes and tumor suppressor genes. In this model, tumors are thought to consist of heterogeneous populations of cells that continue to acquire new mutations, resulting in a highly dynamic process, with clones that outcompete others due to increased proliferative or survival capacity [48]. However, novel insights in cancer stem cell research suggest another layer of complexity in the process of malignant transformation and preservation. It has been reported that only a small fraction of the cancer cells in a malignancy have the capacity to propagate tumors upon transplantation into immuno-compromised mice. Such cells are termed

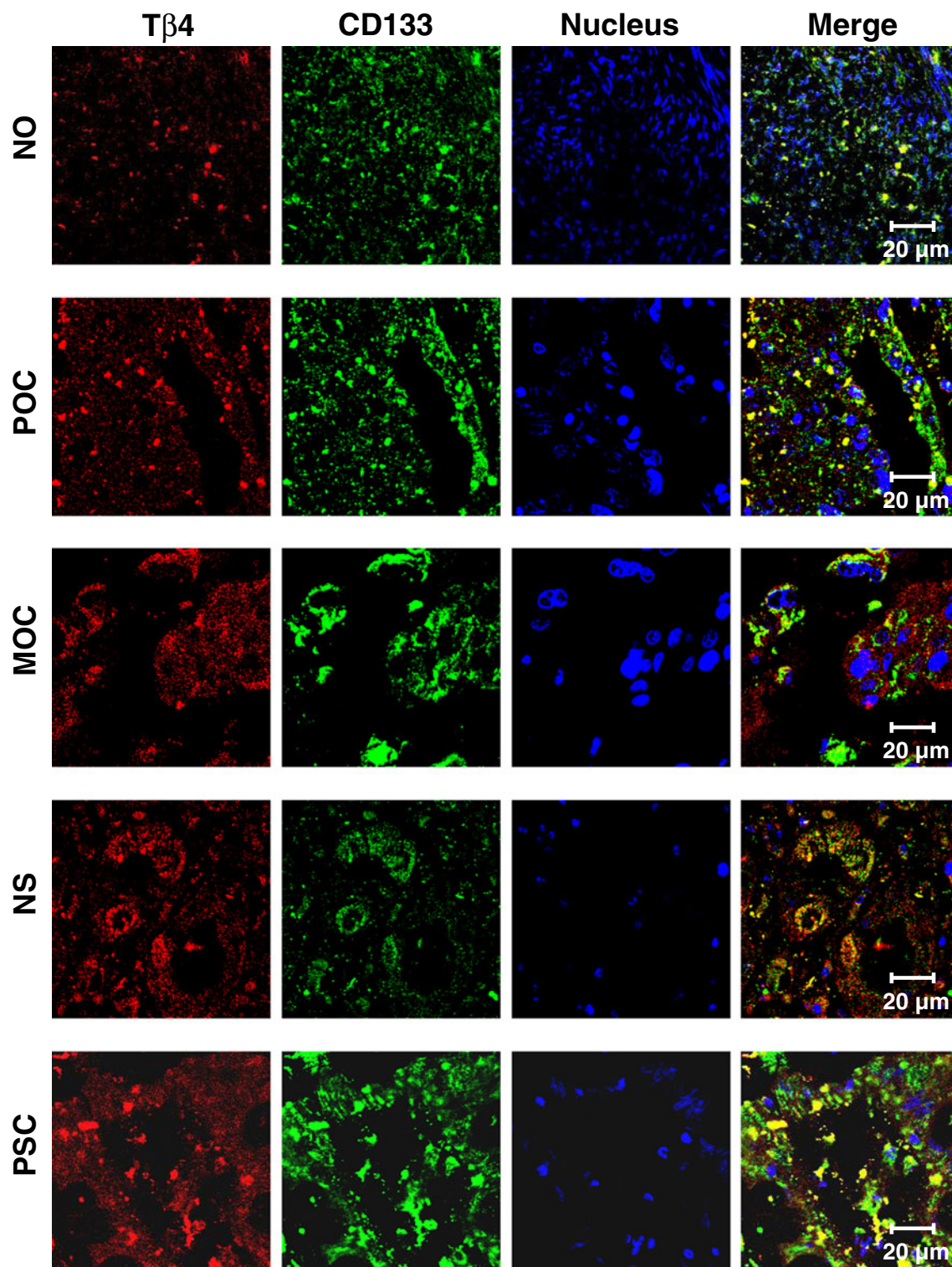


Fig. 3 Co-localization of T β 4 and CD133 in ovarian cancers. Localization of T β 4 and CD133 in ovarian cancers was analyzed in normal ovaries (NO), primary ovarian cancers (POC), metastatic ovarian cancers (MOC), normal stomachs (NS) and primary stomach cancers (PSC) by immunofluorescence staining with double antibodies. Tissue slides were immunostained with mouse monoclonal antibody to T β 4

(1:100) and rabbit polyclonal antibody to CD133 (1:100). After primary antibody incubation, slides were incubated with secondary antibodies (Alexa Fluor 546 anti-mouse antibody and Alexa Fluor 488 anti-rabbit antibodies). The slides were observed for epifluorescence with the use of a confocal laser-scanning microscope

“cancer stem cells” (CSCs) and can be selected based on the expressions of cell surface markers associated with immature cell types [48]. There are a number of approaches to identify cancer stem cell markers, and CD133 has been identified as a powerful cancer stem cell marker [28, 30]. A recent report described colorectal carcinoma cancer stem cells (CR-CSCs) selected by CD133 as the CSC marker and showed that T β 4 promotes several key malignant features of CD133-positive CR-CSCs, ultimately enhancing tumor growth and aggressiveness [44]. T β 4 is weakly expressed in normal colonic epithelial cells, but is considerably up-regulated in CR-CSCs [44]. Furthermore, antisense down-regulation of T β 4 significantly reduced CR-CSC proliferation and cell migration [44].

Here, we first compared the expression pattern of T β 4 and CD133 in ovarian cancers and metastatic ovarian cancers. T β 4 was highly up-regulated in ovarian cancer and metastatic ovarian cancer. The expression of T β 4 in metastatic ovarian cancer from stomach cancer was much higher than that in primary stomach cancer. The expression pattern of CD133 was similar to that of T β 4, showing up-regulation in ovarian cancers and metastatic ovarian cancers and colocalization with T β 4. These data confirm the overexpression of T β 4 in CD133-positive CR-CSCs [44].

The role of T β 4 in tumorigenic activity has been well studied using a lentiviral strategy to downregulate T β 4 expression in CR-CSCs [44]. Down-regulation of T β 4 in CR-CSCs showed a lower capacity to grow and migrate in culture and reductions of tumor size and aggressiveness of CR-CSC-based xenografts in mice [44]. Moreover, loss of tumorigenic activity by downregulation of T β 4 was accompanied by an increase in levels of phosphatase and tensin homologue (PTEN) and a reduction in integrin-linked kinase (ILK) expression, which resulted in a decreased activation of protein kinase B (Akt). Accordingly, exogenous expression of an active form of Akt rescued all of the protumoral features lost after T β 4 targeting in CR-CSCs [44]. Therefore, increases in T β 4 expression in ovarian cancer and metastatic ovarian cancer may be effected through the PTEN, ILK and Akt pathways.

Carcinomas, or tumors derived from epithelial tissues, account for most human cancers. After their initial transformation, cancerous epithelial cells are confined to the primary site by the continued expression of adhesion molecules and the intact basal lamina. However, as cancer progresses, some cells at the periphery of the primary tumor are believed to commandeer the epithelial-mesenchymal transition (EMT) program, thus acquiring mesenchymal properties, which may facilitate the invasion of cancerous cells into both local and distant tissues (metastasis) [49]. T β 4 has recently been proposed to promote the CRC epithelial-mesenchymal transition through the up-regulation of integrin-linked kinase (ILK) [25]. Another study also

suggested that T β 4 is a powerful regulator of EMT in bladder cancer cells [50]. In the present study, we found that T β 4 was up-regulated in both primary ovarian cancers and metastatic ovarian cancers compared with normal controls. These results are consistent with the observation of T β 4 overexpression in ovarian carcinomas [19] and suggest the involvement of T β 4 in EMT of ovarian cancer and stomach cancer.

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