Tumor Endothelial Marker 8 Expression in Triple-negative Breast Cancer

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Abstract. *Background/Aim: Tumor endothelial marker 8 (TEM8) is a tumor endothelial-associated antigen that is having an increasingly recognized role in tumor biology. The expression of TEM8 in triple-negative breast cancer (TNBC) has not yet been characterized. Materials and Methods: We hypothesize that TEM8 is overexpressed in TNBC and in metastatic TNBC in lymph nodes (LN) compared to normal breast tissue and normal lymphatic tissue, respectively. We studied expression of TEM8 in cases of primary (n=17) and metastatic (n=2) TNBC using immunohistochemical analyses. Results: All cases demonstrated increased expression of TEM8 in tumor tissue compared to non-cancerous breast tissue. TEM8 was expressed at a higher level in the stroma adjacent to the TNBC in all cases, with focal immunoreactive areas within the tumor. TEM8 was not expressed in normal lymphoid tissue, but showed expression at sites of LN metastases. Conclusion: TEM8 would appear to represent a new biologic target for designing novel diagnostic or therapeutic approaches for TNBC.*

Breast cancer manifests as a spectrum of clinical behavior defined by the inherent biology of the disease. Triple-negative Breast Cancer (TNBC) is a subtype of breast cancer which lacks protein expression for estrogen receptor, progesterone receptor, and human epidermal growth factor receptor type 2 (HER2) (1-5). TNBC comprises 15% of breast cancer cases and merits intense scientific investigation for its peculiar and often aggressive clinical course. There are currently no targeted therapies available for patients with TNBC and the ultimate development of targeted therapies will rely on identification of biologic targets expressed in TNBC (1).

Tumor endothelial marker 8 (TEM8) is an 85-kDa cellular transmembrane glycoprotein introduced by St. Croix *et al.* in 2000 (6). TEM8 is preferentially expressed in areas of aberrant vessel formation of tumors. It is present in multiple carcinomas including those of breast (7, 8), colon, esophagus, lung, and bladder (6, 9). TEM8 has not been demonstrated to be present in normal tissue such as the corpus luteum, healing wounds, or normal physiologic angiogenesis (6, 9-11), which distinguishes TEM8 from vascular endothelial growth factor (VEGF). The exact physiologic function of TEM8 is unknown but it is thought to play a role in angiogenesis (9), cellular adhesion (12), extracellular matrix homeostasis (13), and promotion of tumor growth (10). It is recognized that TEM8 may be an excellent biologic target for cancer therapy and multiple targeted constructs are being engineered and tested for this purpose (10, 14-16).

Studies investigating the expression of TEM8 in human breast cancer are limited. Davies *et al.* demonstrated that TEM8 is overexpressed in human breast cancer compared to normal breast tissue (7, 8). Further, these authors (7, 8) showed that expression of TEM8 correlates with metastatic disease and/or disease progression. It is noteworthy that in these two prior reports from Davies *et al.*, the expression of TEM8 was not correlated with the expression of the estrogen receptor, progesterone receptor, or HER2. These are receptors which currently help define the biologic behavior of and therapeutic approaches to human breast cancer. Currently, it is unknown whether TEM8 is expressed in patients with TNBC or if TEM8 is expressed at sites of metastatic cancer. The purpose of this study was to characterize the extent and pattern of expression of TEM8 in primary and metastatic human TNBC.

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Key Words: Triple negative breast cancer, TEM8, breast cancer, immunohistochemistry, lymph node metastasis.

Materials and Methods

Seventeen cases of TNBC from Shands Hospital at the University of Florida were analyzed including needle core biopsies and surgical excisions. In all cases, the tumor was negative for estrogen receptor, progesterone receptor, and HER2 expression by immunohistochemistry (IHC). The absence of *HER2* gene amplification was confirmed by FISH (Fluoresce *in situ* hybridixation) in all cases. Immunohistochemistry for TEM8 was performed on all cases according to an established protocol. Briefly, 3- to 4-μm sections of paraffin-embedded, formalin-fixed tissue from each case were mounted on plus slides, dried in an oven at 60˚C, and then stained using an anti-TEM8 antibody (dilution of 1:400; Abcam, Cambridge, MA, USA) in a BenchMark automated immunostainer (Ventana Medical Systems Inc., Tucson, AZ, USA). Heat-induced epitope retrieval was performed with EDTA-buffered cell conditioning (CC1) retrieval solution. Immunostaining was completed using streptavidin-biotin technique and hematoxylin as a counterstain with the iView diaminobenzidine tetrahydrochloride (DAB) detection kit (Ventana Medical Systems Inc.). Appropriate positive and negative controls in each run were acceptable. TEM8 expression was analyzed in all cases by a breast pathologist (SZA). TEM8 expression in TNBC tumor tissue was compared to expression in corresponding normal breast tissue. In two cases with axillary lymph node metastases, immunohistochemistry for TEM8 was also performed on the nodal metastases and corresponding sections of the lymph node without metastasis. The presence of immunoreactivty with TEM8 antibody was estimated by subjectively comparing intensity of immunostaining in cancer cases to that seen in benign breast tissue. A more objective grading system could not be used due to the lack of a common denominator resulting from the natural variation in stromal cellularity and presence of desmoplastic reaction associated with invasive tumor component.

In an attempt to localize the cellular expression pattern of TEM8 in TNBC, dual IHC was also performed according to the procedure described above in the BenchMark automated immunostainer using anti-TEM antibody in combination with one of the following antibodies: calponin (dilution: 1:200; DAKO, Carpinteria, CA, USA), CD34 (pre-diluted; Ventana Medical Systems Inc.), CD31 (dilution: 1:20; DAKO) and α-smooth muscle actin (SMA) (dilution: 1:200; DAKO). The anti-TEM8 antibody immunoreaztivity was detected as brown color using the DAB detection kit, and ummunoreactivity with other antibody simultaneously applied to the same slide was detected as red color using Ultra View Universal Alkaline Phosphatase Red Detection Kit (Ventana Medical Systems Inc.). Approval for this study was obtained from the University of Florida Institutional Review Board.

Results

Immunoreactivity with TEM8 was observed in stromal cells in all cancer cases. Only few scattered immunereactive cells were noted in the stroma in benign breast tissue. In all 17 cases analyzed, the primary TNBC tumor (Figure 1) demonstrated higher expression of TEM8 than normal breast tissue with approximately more than 4-5 times the number of immune reactive cells seen in cancerous tissue compared to benign breast storm. TEM8 was expressed at a higher level in the stroma subjacent to the TNBC and in between tumor cells. None of the TNBC cases showed immunoreactivity for TEM8 in the epithelial tumor cells. Non-specific immunoreactivity was noted in the histiocytes and debris in the necrotic areas of the tumor, and weak non-specific staining in the plasma cells. TEM8 did not appear to highlight any lymphovascular spaces. There was significantly lower expression of TEM8 in the interlobular stroma of benign breast tissue from patients with TNBC (Figure 1B). We observed that TEM8 expression appeared to be higher in cases of TNBC characterized by increased lymphocytic infiltrate (Figure 1D). TEM8 was not expressed in normal lymphoid tissue, but was expressed in the regions with metastatic TNBC within the axillary lymph nodes from two patients (Figure 2). The immunoreactive cells were irregular in shape and some had dendritic processes.

Using dual IHC, the TEM8-positive cells did not show co-expression of calponin, SMA, CD31, or CD34. The anti-calponin antibody only highlighted the intact myoepithelial cell (MEC) layer in normal breast tissue (Figure 3A and B). SMA also highlighted MECs and stromal myofibroblasts which were closely located in relation to the TEM8-positive cells (Figure 3C-E). The anti-CD34 antibody showed the expected immunoreactivity in endothelial cells, which did not show immunoreactivity with anti-TEM8 (Figure 3F-H). Anti-CD31 antibody also highlighted endothelial cells and histiocytes in the stroma (Figure 3I-K). Neither of these cells (endothelial cells/histiocytes) showed co-expression of TEM8.

Discussion

TEM8 is being recognized as a relevant biologic target for cancer therapy due to preferential expression in tumors and not in normal tissues (6, 10, 14, 17). In this report, we have demonstrated for the first time that TEM8 is consistently expressed at higher levels in the stroma of human TNBC compared to benign breast tissue. Furthermore, we also found in a subset of TNBC patients that TEM8 is also expressed at sites of metastatic disease in lymph nodes. TEM8 did not appear to be expressed in the epithelial tumor cells but is overexpressed on cells in the tumor stroma of TNBC.

Dual IHC studies performed on the same tissue section failed to identify the exact nature of the TEM8-positive cells. The TEM8-positive cells do not represent MEC's (calponin- and SMA-positive), endothelial cells (CD34- and CD31-postivie), or histiocytes (CD31-positive, CD34-, and SMA-negative). SMA is a less specific marker than calponin and highlights smooth muscle cells, MEC's, and myofibroblasts. The SMA-positive stromal myofibroblasts were very closely associated with the TEM8-positive cells in the stroma; however, no cells definitively co-expressed both markers.

The lack of immunoreactivity in endothelial cells for TEM8, which were illuminated by routinely used and known vascular markers (CD34 and CD31), observed in this report

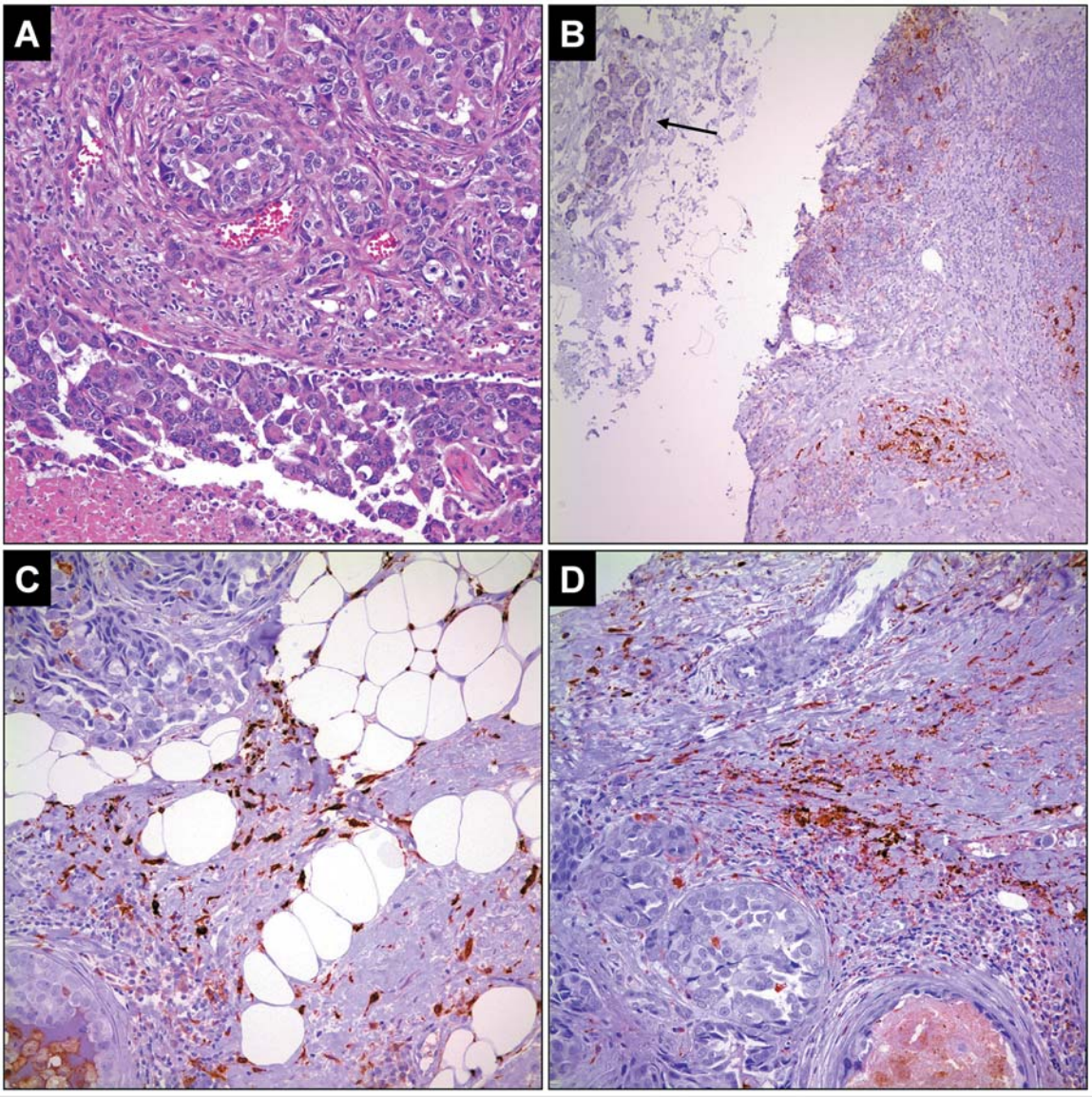

Figure 1. *A: Photomicrography of a representative case of triple-negative breast cancer (hematoxylin and eosin; magnification ×200). B: Biopsy from a patient with TNBC showing preferential expression in the stroma (positive cells appear brown) adjacent to TNBC, while normal breast tissue in the left upper corner (arrow) shows few to no immunoreactive cells in the stroma (IHC TEM8; magnification ×40). C and D: Representative cases of TNBC with high expression of TEM8. Higher staining is noted in (D) where there is more intense lymphocytic infiltrate (IHC for TEM8; magnification ×200).*

differs slightly from previously published work (14). Observed differences may be due to mouse vs. human tissue, or differences in clone and specificity of the antibodies utilized for staining. It is not presently clear if TEM8 is exclusive to one cell type (vascular progenitor cells located in the stroma) or is a product of the tumor microenvironment present on multiple cell types.

In lymphatic tissue, we have observed the expression of TEM8 in lymph nodes where metastatic TNBC cells were present, but not in the normal lymph node tissue. Although some of these TEM8-positive cells in lymph nodes have dendritic processes and may represent dendritic cells, their exact origin is presently unknown. It is interesting to speculate that TEM8-positive cells may be recruited to lymph nodes with cancer cells to support the growth of the metastatic tumor.

The exact function of TEM8 in tumor biology remains unclear although recent work suggests that TEM8 interacts with the actin cytoskeleton and may be involved with cell migration (17). While the mechanism of action is not well

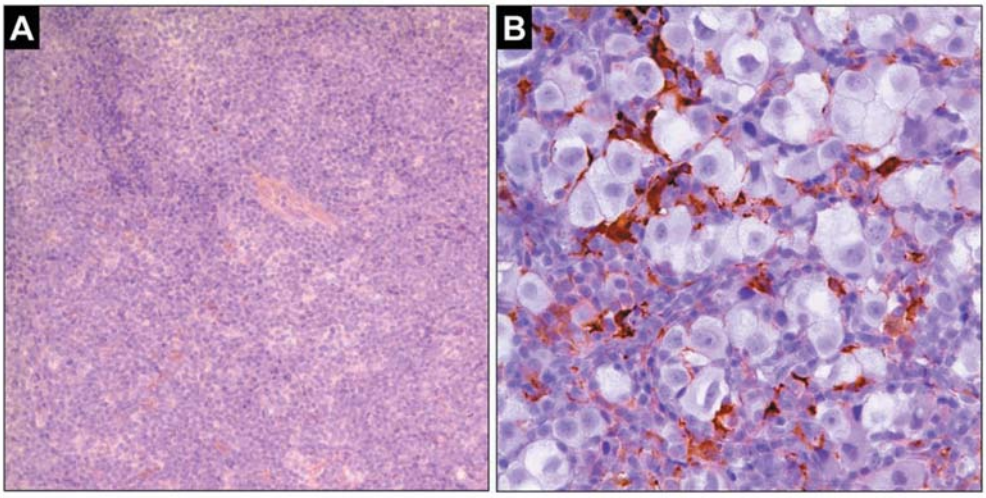

Figure 2. *TEM8 immunohistochemistry of (A) normal lymph node tissue in a patient with metastatic breast cancer to axillary lymph node (magnification: ×200), and (B) lymph node tissue with metastatic triple-negative breast cancer (magnification: ×500).*

understood, there is evidence that blocking TEM8 may be an effective anticancer strategy. Work in mouse xenograft models has demonstrated that targeting TEM8 *in vivo* with a TEM8-Fc fusion protein inhibits growth of breast and colon carcinomas (10). Vaccines against TEM8 in murine models have been effective in slowing tumor growth (15,16). Finally, inhibited growth of melanoma in TEM8 –/– mice has been demonstrated and suggests a role for TEM8 in melanoma growth and progression (13). It remains possible that a TEM8 blockade in human TNBC could have therapeutic value. This is an area which warrants further investigation.

TEM8 has also been used to target anticancer therapeutics to sites of malignancy based on the specificity of TEM8 for tumor tissue and not normal tissues (14). Fernando and Fletcher have demonstrated the antitumor effects of an anti-TEM8/truncated tissue factor fusion protein in a murine xenograft model of colorectal carcinoma (14). It is possible that similar anticancer therapeutics or nanoscale imaging contrast agents could be targeted to primary TNBC using a TEM8-targeting strategy (18). Prior to this, however, one would need to exclude the expression of TEM8 in a variety of non-cancerous breast lesions, including fibroadenomas and papillomas. To our knowledge, TEM8 expression has not been studied in these benign human breast lesions. TEM8 is expressed at sites of metastatic TNBC (Figure 4). The ability to specifically deliver therapeutics to TNBC at metastatic sites using TEM8 as a strategy would represent a major clinical advance.

In conclusion, TEM8 warrants further investigation in TNBC based on the observations made in this report. Future work should be focused on furthering the understanding of the importance of TEM8 in TNBC biology and in tumor progression, better characterizing the expression of TEM8 at sites of metastatic disease, and developing translational strategies for delivery of therapeutics to TNBC using a TEM8-targeted approach. TEM8 may represent a new and important biologic target in TNBC.

Acknowledgements

We would like to thank Ms. Elaine O. Dooley for her technical support and the Florida Biomedical Research Program (Bonkhead Coley) Bridge fund for their financial support.

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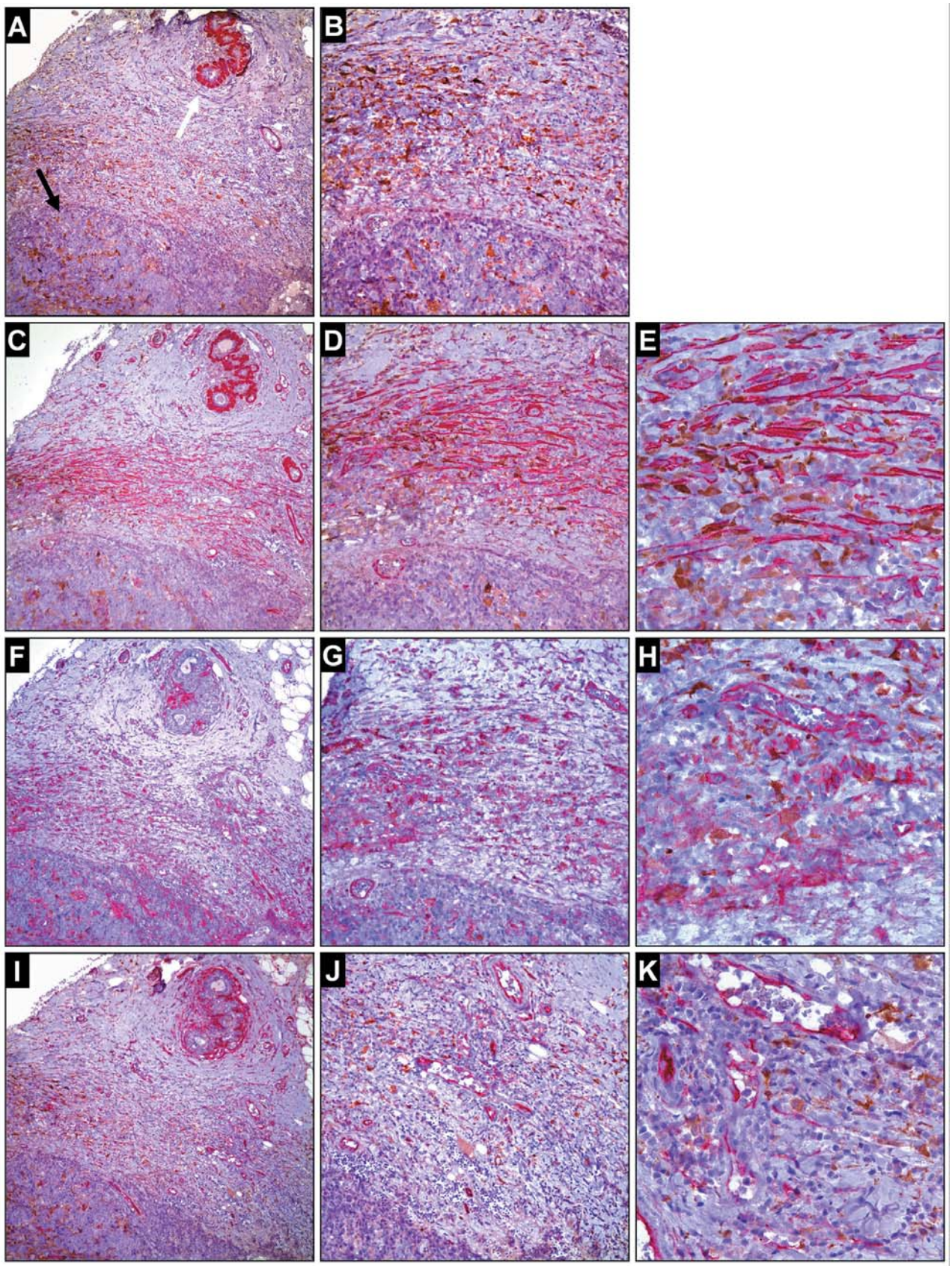
Figure 3. *Photomicrographs of almost the same area of TNBC (black arrow) and adjacent normal tissue (white arrow) with dual (IHC) using anti-TEM8 antibody (in brown) and a second antibody (in red) for calponin (A, B), (SMA) (C-E), CD31 (F-H) and CD34 (I-K). A: Calponin (in red) highlights the intact myoepithelial cell layer in a normal lobule (white arrow) with no staining of the stroma or around the tumor (black arrow). B: Intermediate power view showing TEM8-positive cells in the stroma adjacent to the tumor and in between tumor cells. C: SMA (in red) also highlights the intact myoepithelial cell layer in a normal lobule and stromal myofibroblasts, many of which are closely associated with the TEM8-positive cells (D and E). F and G: IHC for CD31 (in red) and TEM8 in (brown). H: High power view of CD31, highlighting endothelial cells and stromal histiocytes but none of the TEM8-positive cells. Note that the endothelial cells are not immunoreactive for TEM8. I and J: IHC for CD31 (in red), highlighting endothelial cells and TEM8 (in brown). K: High power view of CD34, highlighting endothelial cells but not stromal histiocytes or TEM8-positive cells. Note that the endothelial cells are not immunoreactive for TEM8. (magnifications: A, C, F and I : ×100, B,D, G and J: ×200, E, H and K: ×500).*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Conflict of Interest

All Authors confirm no conflict of interest.

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Received July 15, 2011
Revised September 12, 2011
Accepted September 13, 2011