Original Article

A comparative immunohistochemical analysis of cathepsins B and S in human breast cancer

Aula Ammar, Maram Blal, Amani Halabi, Zuheir Al-Shehabi[1]

Department of Biochemistry and Microbiology, School of Pharmacy, [1]Department of Pathology, School of Medicine, Tishreen University, Latakia, Syria

ABSTRACT

Background: Cancer progression is a complex process consisting of a series of distinct steps. Cysteine proteases, such as cathepsins (Cts), are important molecules that play a central role in cancer progression and metastasis. Previous studies on human and mouse models of pancreatic cancer showed that both Cts B and Cts S are highly expressed in malignant tissues and the infiltrating macrophages. The aim of this study was to investigate the expression pattern of Cts B and S in human breast cancer tissues. **Materials and Methods:** Twenty-three formalin-fixed paraffin-embedded sections of breast cancer were stained for Cts B, Cts S, and CD206 using immunohistochemistry. **Results:** Cytoplasmic staining of Cts B and S was observed in tumor cells, endothelial cells, and macrophages. Cts B was preferentially expressed in breast cancer tissues by the different cells types. The majority of tumor samples were Cts B-positive in tumor cells, endothelial cells and macrophages (91%, 87%, and 70%, respectively) in comparison to Cts S (39%, 48%, and 57%, respectively; $P < 0.001$, $P < 0.001$ and 0.002). Correlation studies indicated significant relationships between the vascular and macrophage expression of Cts B ($P = 0.01$) and of Cts S ($P = 0.03$). However, neither Cts B nor Cts S expression in tumor cells correlated with other cell types ($P > 0.05$). Only the expression of Cts B in vascular endothelial cells correlated significantly with the tumor grade ($P = 0.03$). **Conclusion:** Results suggest that Cts B expression is more prominent than Cts S in breast cancer. Correlation studies imply different mechanisms regulating Cts B/S expression in tumor cells and other stromal components.

Key words: Breast cancer, cathepsin B, cathepsin S, tumor-associated macrophages

INTRODUCTION

Cancer is a hyperproliferative disorder, which is initiated by mutations in key regulatory genes, and then changes in protein function occur.[1-3] Cancer cells undergo morphological transformation, dysregulation of apoptosis, and uncontrolled proliferation, and later have the ability to invade and metastasize.[4] Breast cancer is the most common malignancy and the leading cause of cancer-related death in women worldwide.[5] During tumorgenesis, cancer cells produce many soluble mediators that recruit and activate inflammatory cells.[1] It has been considered that such infiltration of inflammatory cells may be an evidence of the host response to the tumor.[6] Moreover, inflammation is associated with the initiation and promotion of specific cancers such as colorectal, gastric, liver, and breast.[2]

The inflammatory infiltrate produce cytokines and proteases that enhance tumor growth, invasion and metastasis. Among these cells, tumor-associated macrophages (TAMs) are frequently the most prominent leukocytes present in a tumor.[1] Most TAMs are considered Type-2, or alternatively activated, macrophages which promote a suppressed immune response against the malignancy, produce factors involved in tissue remodeling (matrix proteins) and angiogenesis.[7,8] Both TAMs and tumor cells express different groups of proteases, including matrix metalloproteinases (MMPs), serine proteases, and cathepsins (Cts), which lead to extracelluar matrix proteins degradation and tumor invasion.[9,10]

Cysteine Cts are a subgroup of the Cts family. They include approximately 11 members usually referred to using letters (e.g., Cts D, B, S, X, L, etc.). Cysteine Cts are secreted from leukocytes, such as macrophages and lymphocytes, and from myoepithelial cells.[11] The important roles of these Cts in tumor progression, angiogenesis, invasion,

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Address for correspondence: Dr. Aula Ammar, Department of Biochemistry and Microbiology, School of Pharmacy, Tishreen University, Latakia, Syria. E-mail: aulaammar@yahoo.com

and metastasis are well-established.[3] For example, Cts D facilitates the release of basic fibroblast growth factor from the extracellular matrix, which induces angiogenesis in breast cancer.[12] Furthermore, the significant increase in total Cts D and pro-Cts D in the serum of patients with metastatic breast carcinoma suggests Cts D as a poor prognostic marker for metastasis.[13] Different studies supported similar effects of other members of the Cts family in different tumor models. It has been shown that removing Cts B or S in mice model of pancreatic cancer reduced the frequency of angiogenic switch and subsequently the development of the tumor vasculature and tumor growth.[14] Moreover, the deletion of Cts B/S genes from macrophages significantly reduced tumor invasion *in vivo*; on the other hand, restoring the expression of these proteases regenerated invasiveness to wild-type levels.[15] Such significant findings about the role of concomitant presence of Cts B and S in mice models of pancreatic and breast cancer has not yet been addressed in human breast tumors. Therefore, the aim of this study is to assess the expression of Cts B and S in human breast cancer tissues and correlate that with the tumor grade.

MATERIALS AND METHODS

Breast cancer specimens

Twenty-three formalin-fixed paraffin embedded breast cancer specimens were used in the analysis. Specimens were obtained from the Department of Histopathology. A written consent was signed by every patient and approved by the Institutional Ethical Board. The mean age of patients at the time of diagnosis was 53.5 years (32-75 years). The mean size of tumors was 4.8 cm (1.5-8 cm). The histological grade of tumors was: Grade I (n = 2, 8.7%), Grade II (n = 9, 39.1%), Grade III (n = 8, 34.8%). For technical reasons, the tumor grade of four patients was missing. Approximately, 44% (n = 10) of the tumors were invasive ductal carcinoma and 39% of the samples were of the invasive lobular type of tumor.

Immunohistochemical staining

Serial sections (5 μm thick) were prepared from each block. For immunohistochemistry staining, sections were deparaffinized in xylene and dehydrated in ethanol baths (100-30%) then in distilled water. For blocking endogenous peroxidase, sections were incubated for 20 min in 3% hydrogen peroxide in methanol. Antigen retrieval was performed in hot citrate (pH = 6, 60°C) for 1 h. Background sniper (Biocare Medical, California, USA) was used to block nonspecific binding, followed by adding the primary antibodies against Cts B or S (1:50, R and D systems, USA), anti-CD206 (1:20, R and D systems, USA), CD68 (1:20, R and D systems, USA) at 4°C overnight. CD68 was used as a positive control for macrophages in tonsils and not as part of the analysis of tumor sections. Primary antibodies were washed using tris buffered saline (TBS), then incubated with horseradish peroxidase (HRP)-conjugated anti-goat antibody (1:500, R and D systems, USA) for 1 h at room temperature or with the 4 plus HRP universal detection (Biocare Medical, California, USA) according to the manufacturer instruction. After washing in TBS, sections were incubated for 1 min with diaminobenzidine (DAB, Biocare Medical, California, USA), counterstained in hematoxylin, dehydrated and mounted using Canada balsam. Tonsil tissues were used as positive controls for Cts expression. Negative controls were stained using the same procedure, but excluding the primary antibody.

Immunohistochemical analysis

The expression of Cts B and Cts S in tumor sections was assessed separately in tumor cells, endothelial cell and infiltrating leukocytes. Each section was evaluated as negative (0) when no positive cells were observed, weak (+1) when <30% of cells were positive, moderate (+2) when 30-60% of cells were positive, and strong (+3) when more than 60% of cells were positive.[16] The score given to the section represents the average of the area of expression in ten microscopic fields examined at magnification × 400. The density of CD206-positive macrophages was categorized into three groups: No staining (0), low (1) and high (2). All samples were examined by two independent assessors, blinded to tumor data, with reanalysis of any discrepancies.

Statistical analysis

The expression of Cts B and Cts S was classified into four categories (0, 1, 2, and 3) according to the percentage of positive staining. CD206 positive cells were categorized in three groups (0, 1, and 2). Using cross tables and Chi-squared test, the relationships between categorized data of Cts B and Cts S expression in tumor/endothelial/immune cells and the tumor grade and between the Cts B expression by the different cell types and CD206 classified data were assessed. $P \leq 0.05$ defined a significant relationship. Statistical analysis was performed using SPSS, version 18.0 (SPSS Inc, USA).

RESULTS

Cathepsins B and S are expressed in macrophages and endothelial cells of tonsils and tumors and also in tumor cells

By using antibodies against Cts B, Cts S, and CD68 on tonsil sections, cytoplasmic staining was present in macrophages of the germinal centers [Figure 1a-c] and in the vascular endothelial cells [Figure 1d]. Both Cts B and S were also expressed in the cytoplasm of tumor cells, endothelial cells, and other stromal cells [most probably macrophages, Figure 1e and f]. Occasional weak staining was observed in the normal ductal epithelium of the tumor periphery (data not shown).

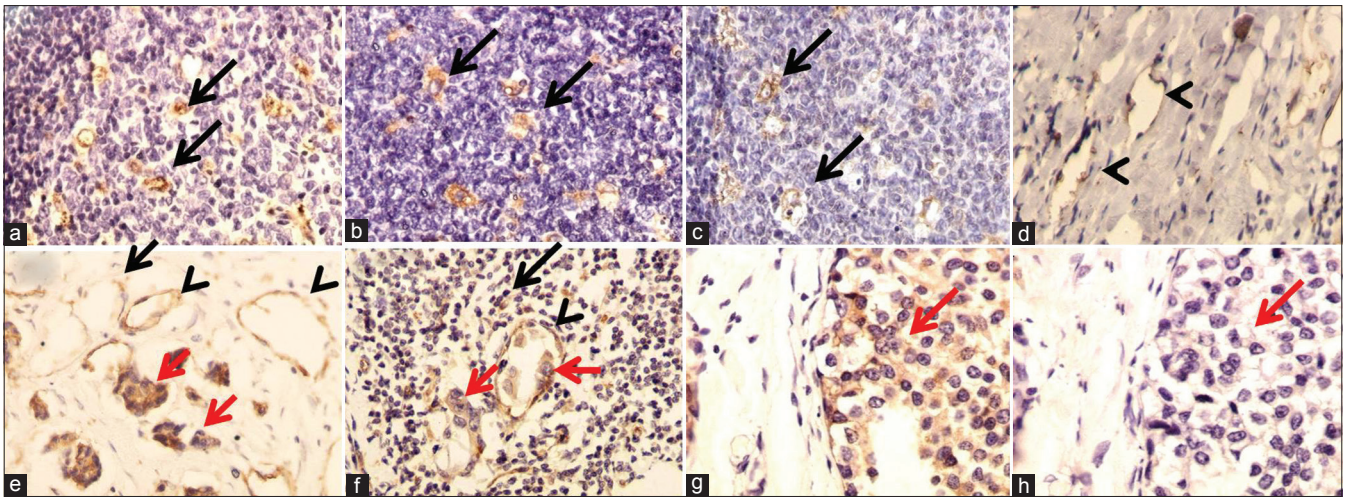

Figure 1: Expression of cathepsins (Cts) B and Cts S in tonsils (a-d) and formalin-fixed paraffin embedded breast tumors (e-h). Macrophage (black arrows) stained positive for Cts B (a) and S (b). (c) The pan macrophage marker CD68. (d) An example of a positive Cts B staining in tonsil vessels (arrow heads); similar staining was observed with Cts S in vessels (not shown). Tumor cells (red arrow) and tumor vasculature (arrow heads) and macrophages (black arrows) express Cts B and S (e and f, respectively). (g and h) An example of Cts B-positive/Cts S-negative tumor cells (a-h, ×400)

Breast cancer tissues preferentially express cathepsins B rather than cathepsins S

Although the cellular expression patterns of Cts B and S were similar, the percentages of samples expressing Cts B in all cell types were higher than those expressing Cts S, and these differences were statistically significant. In the current study, the majority of breast cancer tissues (91% [21 samples]; including all samples scored 1, 2, or 3) express Cts B in tumor cells compared to 39% samples positive to Cts S [$P < 0.001$, Table 1]. Figure 1g and h shows one representative example of a positive Cts B staining versus a negative Cts S staining in the same tumor specimen. Endothelial cells lining vessels also stained positive for Cts B in 87% of samples compared to 48% with Cts S [Table 1] ($P < 0.001$). TAMs showed positivity to Cts B and S in 70% and 57% of specimens, respectively and this difference was statistically significant [$P = 0.002$; Table 1].

The distribution of tumor samples within the specified categories according to the area of expression (0, 1, 2, and 3) in the different cell types was further assessed. Figure 2 shows the percentages of samples expressing Cts B and S in the different groups and the various cell types. In tumor cells, Cts B expression was distributed among the three categories 1, 2, and 3 (39%, 13, and 39%, respectively), whereas the majority of samples were negative to Cts S in tumor cells (61%), and Cts S expression was always weak when present (score 1) [Figure 2a]. Figure 2b represents the differences among percentages of scored samples for Cts B and Cts S in vessels, and these percentages reveal opposite trends; that is, Cts B staining in vessels seems to be proportional to the score given (percentages were 13%, 17%, 26%, and 44% for scores 0, 1, 2, and 3, respectively) [Figure 2b]. On the other hand, most Cts S-positive vessels in tumor sections scored 0 and 1 (52% and 35%, respectively) [Figure 2b].

Table 1: Percentage of positive tissues for Cts B and Cts S according to the type of positive cells

Cell type	Positivity to Cts (%)		*P* value
	B	S	
Tumor cells	91 (*n*=21)	39 (*n*=9)	<0.001
Endothelial cells	87 (*n*=20)	48 (*n*=11)	<0.001
Stromal cells	70 (*n*=16)	57 (*n*=13)	0.002

Cts: Cathepsins

Scores and percentages of Cts expression in macrophages are shown in Figure 2c. Approximately, one-third of tumors were negative for Cts B in macrophages compared to 44% negative with Cts S. Approximately, 35%, 26%, and 9% of cases scored 1, 2, and 3, respectively for Cts B in macrophages, whereas 52% of the cases scored 1 for Cts S in macrophages and the rest scored 2 with none scored 3 [Figure 2c].

It was also interesting to explore the relationship between the expression of each Cts in the different cell types (tumor cells, vessels, and macrophages). Correlation studies using Pearson Chi-squared test showed a significant association between Cts B expression in vessels and macrophages ($P = 0.01$); yet Cts B expression in endothelial cells/macrophages did not correlate with the tumor cells ($P > 0.05$). Similarly, the correlation between Cts S expression in vessels and macrophages was also considerable ($P = 0.03$), but no other significant correlations between other cells types were present ($P > 0.05$).

The associations between Cts B and S expression in the different cell types (tumor cells, vessels, and macrophages) and the tumor grade were also analyzed, and results are shown in Table 2. There were no significant relationships between either Cts B or S expression in tumor cells or macrophages and the grade of the tumor [Table 2].

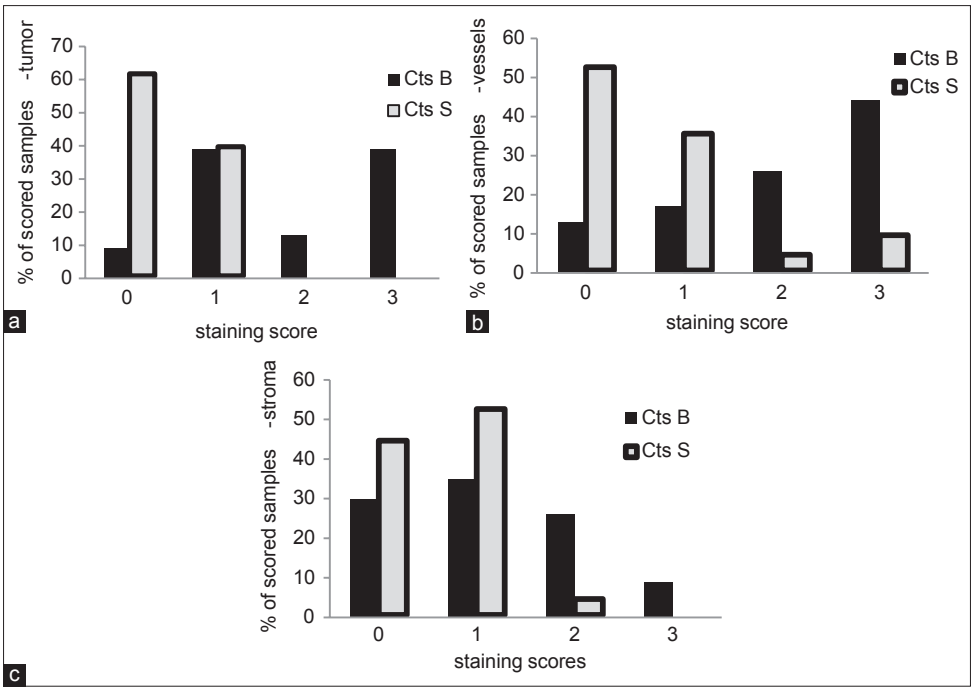

Figure 2: The percentages of the expression categories of cathepsins (Cts) B and Cts S in tumor cells, vessels, and macrophages. The percentages of samples categorized in four groups 0, 1, 2 and 3 are shown in tumor cells (a), vascular endothelium (b) and macrophages (c)

However, Cts B level in vessels associated with the tumor grade ($P = 0.030$). No similar significance was seen between the vascular expression of Cts S and the tumor grade [$P = 0.027$, Table 2]. These results suggest a pro-angiogenic role of Cts B in breast cancer.

Type-2 macrophages express cathepsins B in breast cancer tissues

To identify whether Type-2 macrophages are also involved in Cts proteases expression, tumor sections were stained with anti-CD206, which specifies Type-2, or alternatively activated, macrophages. Results showed that CD206 positive macrophages infiltrate tumor stroma and surround necrotic areas. Staining also revealed a co-expression of CD206 and Cts B mainly in the periphery of the tumor [Figure 3a-d]. CD206 was only compared to Cts B because its expression was superior to Cts S in breast cancer tissues.

A significant association was present between CD206 expressing-cells and Cts B in vessels [$P = 0.004$, Table 3]. However, no similar correlation was observed with other types of Cts B-positive tumor cells ($P > 0.05$, data not shown).

Table 2: Correlation of Cts B or Cts S expression in different cell types with the tumor grade

Cts B expression	I	II	III	*P* value	Cts S expression	I	II	III	*P* value
Tumor cells					Tumor cells				
0	0	1	0	0.47	0	2	6	3	0.21
1	0	3	4		1	0	3	5	
2	1	2	0		2	0	0	0	
3	1	3	4		3	0	0	0	
Endothelial cells					Endothelial cells				
0	2	1	0	0.03	0	2	6	3	0.27
1	0	2	2		1	0	2	5	
2	0	3	1		2	0	0	0	
3	0	3	5		3	0	0	0	
Stromal cells					Stromal cells				
0	1	3	2	0.93	0	2	5	3	0.39
1	1	2	3		1	0	3	5	
2	0	3	2		2	0	1	0	
3	0	1	1		3	0	0	0	

Cts: Cathepsins

Table 3: Correlation between CD206 expression in macrophages and Cts B in vessels

Cts B staining in vessels (%)	CD206-macrophages			*P* value
	Non	Low	High	
0 (none)	2	1	0	0.004
1 (<30)	0	4	0	
2 (30-60)	6	0	0	
3 (>60)	1	4	3	

Cts: Cathepsins

DISCUSSION

Proteases have been implicated in the process of tumor invasion and metastasis, including MMPs, serine proteases, and Cts.[9] Different cell types within the tumor environment, such as tumor cells, infiltrating immune cells, endothelial cells, myoepithelial cells and fibroblasts, may secrete proteases. The role of some members of the Cts family, such as Cts D, has been well-characterized. It has been suggested that Cts D is particularly important in the progression of human breast cancer.[17,18] However, the role of other members of the Cts family has since been

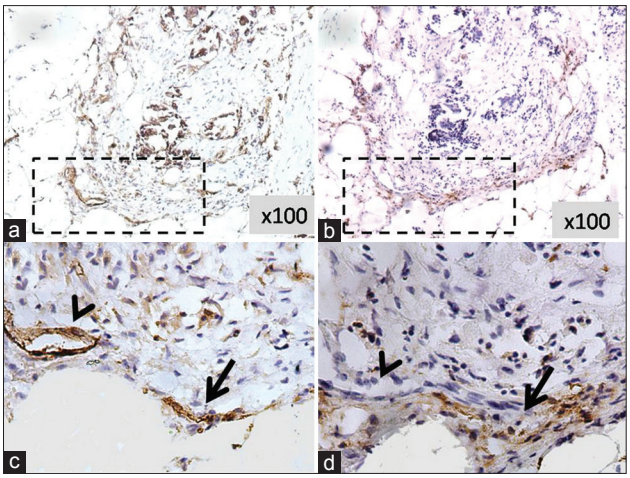

Figure 3: Cathepsins B (Cts B) expression in CD206 positive macrophages. Peripheral staining of tumor stroma with Cts B (a and c) and CD206 (b and d). (a and b, ×100; c and d, ×400). Arrows refer to macrophages, arrow heads indicate vessels

under investigation to assess their involvement in tumor progression and/or metastasis. Of these Cts, Cts B, S, and L are cysteine proteinases that have been correlated with aggressive tumor behavior in mice model of pancreatic and mammary cancer and in human pancreatic cancer.[14,15] To the best of our knowledge, there has been no study comparing Cts B and S expression in human mammary tumors. Therefore, the current study aimed to compare the expression pattern of Cts B and S in human samples of breast cancers, and to correlate their expression with the tumor grade.

The results showed that both Cts B and Cts S are expressed in tumor cells, endothelial cells and infiltrating leukocytes [most probably macrophages, Figure 1]. Previous studies reported similar distribution pattern of Cts B in breast and brain cancer.[16,19] Castiglioni *et al*.[16] found that human breast cancer tissues express Cts B in tumor nests and macrophages. In the study by Strojnik *et al*. on the human brain tumors,[19] it has also been observed that Cts B is present in brain tumor cells, endothelial cells, and macrophages. Furthermore, the co-expression of Cts B, L, and S was also investigated by Gocheva *et al*. in a study on murine models of pancreatic cancer and human pancreatic endocrine neoplasms tissues.[14] Gocheva *et al*. found that in an animal model of pancreatic cancers Cts B, L, and S were expressed by both tumor cells and endothelial cells; in human pancreatic tissues, the results were slightly different.[14] Cts B expression in human pancreatic cancers was in accordance with the murine models, yet Cts S was either absent or expressed at low levels in human tissues. Gocheva *et al*. attributed this discrepancy in Cts S expression between mouse and human pancreatic cancer to the different methods of tissue fixation.[14] Results of the current study revealed that breast cancer tumors preferentially express Cts B rather than Cts S in the different cell types (i.e., tumor cells, vessels, and macrophages), and the observed difference was statistically significant [Table 1]. Such results potentially imply a significant role of Cts B rather than Cts S in breast tumors, yet Cts S may still play a role in the tumorigenesis of other cancer types.

The regulation of Cts protease expression and activation in the tumor environment is poorly understood. For example, cytokines such as interleukin 4 (IL-4) may be involved in TAM-derived Cts B and S activation in pancreatic cancer.[15] Other mechanisms may also play a role in Cts activity on different levels that is, transcription, translation, posttranslational modifications, maturation, trafficking, and inhibition (reviewed in[11]). In general, the regulation of Cts activity seems to be a complex process, which may follow different mechanisms in different cell types. In this study, there was a significant association between the expression of Cts B in vessels and macrophages, but none correlated with its expression in tumor cells. Similar observations were reported with Cts S expression. Since, we found that the expression of Cts in stromal component (vessels and macrophages) is correlated with each other and not with tumor cells; this may indicate that the expression of Cts B or Cts S in stromal cells may respond to different stimulants from tumor cells. Further *in vitro* work investigating the role of different stimuli, such as cytokines such as IL-4, on Cts B and S expression in various representative cell models is necessary.

Tumor vasculature express Cts B and S yet Cts B seems to be more prominent in the tumor-associated endothelia [Table 1, $P < 0.001$]. The significant association between Cts B in vessels and the tumor grade [$P = 0.03$, Table 2] supports the angiogenesis-promoting role of Cts B in human pancreatic cancer.[14]

The lack of statistical significance between Cts B expression in tumor cells and the grade [Table 2] may be explained by Cts B role in the invasive properties of tumor cells rather than the histological grading (malignant transformation of mammary cells from low to high grade). Therefore, it would be expected to find significance with the stage (which includes tumor size, lymph node metastasis, and the distant metastasis). In accordance with this explanation, previous studies reported a significant relationship between Cts D or B and the tumor stage.[16] Therefore, it will be interesting to study the relationship between Cts B and the clinical stage of tumors.

Tumor-associated macrophages seem to play a key role in tumor progression.[20] Both TAMs and tumor cells express several proteases that have been implicated in the process of tumor invasion and metastasis, including MMPs, serine proteases, and Cts.[9] It has been suggested that Cts D,

B, S, and L are particularly important in the progression of human breast cancer and other mouse models of cancer.[17,18] Most of the previously published data used pan macrophage markers such as CD68 to assess macrophages. In the current study, the question about the secretion of Cts B from a vital subpopulation of macrophages, the Type-2 macrophages, was addressed. CD206-positive macrophages correlated with Cts B expression in vessels rather than in tumor cells [Table 3], supporting the idea discussed above about the possibility of common regulatory mechanisms controlling Cts expression in the tumor stroma.

Finally, although the patient sample used in this study is small and is considered as a limitation for the statistical analysis conducted, further studies should include a larger number of patients in order to verify the statistical results. Yet in the current study, our immunohistochemical analysis demonstrated that Cts B, and S are localized in tumor cells, stroma, and endothelial cells of human breast cancer. We showed an increased expression of Cts B in a significant percentage of tumor samples as compared with Cts S. The expression of Cts B and S within the tumor environment may be controlled by different mechanisms in the cellular components (i.e., tumor versus stromal cells). Cts B may be a key player in tumor angiogenesis, and its expression in the tumor vasculature is associated with the tumor grade. Understanding the regulation of Cts B expression in breast tumors will help in finding novel potential targets for cancer treatment.

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