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Cathepsin S and its inhibitor cystatin C: imbalance in uveal melanoma

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1. ABSTRACT

The present study aimed to investigate, as a follow-up of microarray profiling, the expression of the lysosomal cysteine protease cathepsin S and that of its endogenous inhibitor cystatin C in the most common primary intraocular tumor in adults, uveal melanoma. The expression pattern unveiled was characterized by a relative increase in the active form of the elastolytic and collagenolytic cathepsin S that was not counterbalanced by the expression of its strongest endogenous inhibitor cystatin C in the aggressive, highly metastatic uveal melanomas. The study provides evidence for a novel correlation between a specific cysteine protease activity and the strongest predictive factor for metastatic behavior in primary uveal melanoma and documents the first investigation of both a specific protease activity and its endogenous inhibitor in uveal melanoma. The results indicate that the shift in the balance between cathepsin S and cystatin C may be part of deregulated proteolytic pathways contributing to the invasive phenotype of uveal melanoma.

2. INTRODUCTION

Cysteine cathepsins and their endogenous inhibitors have been implicated in various processes related to initiation and progression of cancers, such as tumor growth, invasion, remodeling of tumor microenvironment, angiogenesis, inflammation and apoptosis (1-3). Furthermore, the ability of tumor cells to metastasize has been associated with increased or deregulated cysteine-proteolytic enzyme activity (4-6). Cathepsins, lysosomal cysteine proteases that are distinguished by their substrate specificities as well as by their mechanism of action (7), have been found to be upregulated in many human tumors including pancreas (8), ovarian (9), breast (10), prostate (11) and skin melanoma (12). Increased cysteine cathepsins expression in tumors has been attributed to multiple mechanisms, including gene amplification, alternative splicing and post-translational modifications (13). However, the functions of these enzymes and of their specific inhibitors in tumor cells and tumor-associated processes are not well defined.

Recent data from microarray-based molecular profiling of primary uveal melanoma specimens suggested that cathepsin S has a variable pattern of expression in aggressive, highly metastatic uveal melanomas (characterized by monosomy 3 genotype) compared to less aggressive, disomy 2 melanomas, while cystatin C, the most potent endogenous cysteine proteinase inhibitor is among the non-changing genes at transcriptional level (14). In the light of this data, the present study aimed to investigate at protein level the characteristics of cathepsin S and cystatin C expression in uveal melanomas of different cytogenetical and pathological features.

Melanoma of the uveal tract is the most common intraocular primary tumor in adults (15). Most uveal melanomas develop from melanocytes of the choroid and fewer arise in the ciliary body or iris (16). Metastases involve preferentially the liver and occur, due to the absence of intraocular lymphatics, through hematogenous dissemination (15). Characteristics most significantly associated with poor prognosis include partial or complete loss of chromosome 3 (monosomy 3) with or without partial gains in chromosome 8, ciliary body involvement, large basal tumor diameter, scleral invasion, presence of epithelioid-type cells and closed Periodic Acid Schiff (PAS)-positive loops/microcirculation patterns (17-21). Monosomy 3 in primary tumor cells constitutes the most common genotypic aberration in uveal melanoma (22) and has the strongest association with poor prognosis of all the clinico-pathological factors studied to date (23, 24).

Cathepsin S is an elastolytic lysosomal cysteine endoprotease that has a more limited expression compared with the other cathepsins, being specific to the spleen and immune cells (25). The expression of cathepsin S in antigen presenting cells, dendritic cells and macrophages is consistent with the role of this enzyme in class II-mediated antigen processing and presentation (26). In addition to the role in the MHC class II immune response, cathepsin S has been attributed a role in extracellular proteolysis including elastin degradation and collagen processing (27), general lysosomal proteolysis with specific implications in angiogenesis (28) and atherosclerosis (29, 30). In the eye, cathepsin S proteolytic activity has been implicated, either directly or through the regulation of cathepsin D activity, in the process of degradation of photoreceptor outer segment by retinal pigment epithelial cells (31, 32). Unlike most cathepsins, which are unstable and weakly active at extracellular neutral pH, cathepsin S has strong elastinolytic activity even at neutral pH (33, 34), a property that specifically enables it to play an extracellular role.

Cystatin C is a biochemically well-characterized, strong inhibitor of cysteine proteinases and also of some lysosomal caspases. It is widely expressed by various human tissues and present at physiological concentration in extracellular fluids (35). The inhibitor is expressed as a precursor which is processed through the secretory pathway of the cells and thereby secreted as the active, mature form with a monomeric molecular mass of approximately 13 kDa (36, 37). The broad expression pattern and inhibitory activity of cystatin C indicate multiple and varied roles that are most likely cell or tissue-specific. Involvement in bone resorption (38), remodelling of the extracellular matrix in the arterial wall (29), modulation of neutrophil chemotactic activity (39) and regulation of neuronal, oxidative stress-triggered apoptosis (40) are among the biological roles suggested for cystatin C. In the eye, the two main sites of expression of cystatin C in non-pathological conditions are the ciliary epithelium, which most likely accounts for the presence of the inhibitor in the aqueous, and the retinal pigment epithelium (35). The function of cystatin C secreted constitutively by the retinal pigment epithelium, the cellular monolayer separating the choroid from the neuroretina, appears to be associated with extracellular processes taking place at the basal side of the epithelium (36).

Here we report the findings of the first investigation into the expression of specific molecular determinants of proteolytic activity in uveal melanomas. Our results show that the increase in the proteolytic activity resulting from an imbalance between a specific cysteine protease, cathepsin S, and its endogenous inhibitor, cystatin C, associates with aggressive, highly metastatic uveal melanomas. The findings are relevant for the prospect of developing anti-cancer strategies based on targeting cysteine cathepsins activity, in addition to understanding the (dys)regulation of proteolytic activity in uveal melanoma.

3. MATERIALS AND METHODS

3.1. Tumor specimens

Tissue lysates of 40 primary uveal (choroidal) melanoma specimens were used for immunoblotting. The tumor specimens were from patients (22 males and 18 females) with a mean age at treatment of 62 years, SD±12 years, treated by enucleation (38 cases) or local resection (2 cases) at the Ocular Oncology Centre, Royal Liverpool University Hospital between 2002 and 2004. Dissected tumor specimens were snap frozen in liquid nitrogen in the operating theatre and stored at -80°C. Additionally, sections from 24 randomly selected formalin-fixed, wax-embedded tumor-containing whole eye globes were used for immunohistochemical staining. Diagnosis was confirmed by pathological examination of the specimens. Histopathological and cytogenetical parameters (including ciliary body involvement, scleral/extraocular invasion, tumor size, cell type, presence of periodic acid-Schiff (PAS)-positive loops and status of markers of chromosomes 3 and 8) were recorded for all tumor specimens. Informed consent was obtained from patients and the study was approved by the Liverpool Research Ethics Committee.

3.2. Western immunoblotting analysis

Each primary tumor tissue specimen was homogenized in PBS containing protease inhibitor cocktail (Sigma-Aldrich Company Ltd, Dorset, UK) and samples were resolved by electrophoresis in 10% sodium dodecyl sulphate (SDS)-polyacrylamide gels prior to transfer to nitrocellulose membranes (Schleicher & Schuell® Protron®, pore size, 0.45 μm; Sigma Aldrich Company

Table 1. Antibodies and optimized conditions for immunoblot analysis

Primary antibody	Dilution	Secondary antibody (dilution)
Anti-Cathepsin S polyclonal IgG[1]	1:500	HRP-conjugated rabbit anti-goat IgG (1:1000)[4]
Anti-Cystatin C polyclonal IgG[2]	1:500	HRP-conjugated goat anti-rabbit IgG (1:000)[4]
Anti-GAPDH monoclonal (6C5)[3]	1:2000	HRP-conjugated goat anti-mouse IgG (1:2000)[4]

IgG, immunoglobulin G; HRP, horseradish peroxidise; GAPDH, glyceraldehyde phosphate dehydrogenase. [1] Source: R&D Systems Europe Ltd., Abingdon, UK. [2] Source: Upstate Biotechnology, Millipore (UK) Ltd, Dundee, UK. [3] Source: Abcam Plc, Cambridge, UK. [4] Source: Sigma Aldrich Company Ltd, Dorset, UK.

Table 2. Optimized conditions for cathepsin S and cystatin C immunostaining analysis

Primary antibody	Dilution (incubation)	Secondary antibody (dilution;incubation)
Anti-Cathepsin S polyclonal IgG[1]	1:20 (1 hr, RT)	Biotinylated rabbit anti-goat IgG[3] (1:200; 30min, RT), followed by HRP-streptavidin[3] (1:500; 30 min, RT)
Anti-Cystatin C polyclonal IgG[2]	1:200 (overnight, 4°C)	REAL™ EnVision™ HRP-mouse/rabbit dual-linked polymer[4] (30 min, RT)

IgG, immunoglobulin G; RT, room temperature; HRP, horseradish peroxidise. [1] Source: R&D Systems Europe Ltd., Abingdon, UK. [2] Source: Upstate Biotechnology, Millipore (UK) Ltd, Dundee, UK. [3] Source: Vector Laboratories UK, Peterborough, Cambridgeshire, UK. [4] Source: Dako UK Ltd, Ely, Cambridgeshire, UK. All primary antibody incubations were followed by three washes in 1% rabbit, respectively goat, normal serum in PBS/0.05% Tween 20. Incubations with the respective secondary antibodies were followed by three washes with PBS/0.05% Tween 20.

All incubations with primary antibodies were performed overnight at 4°C and followed by incubation with respective secondary antibodies of 1 hour at room temperature.

Ltd, Dorset, UK). Precision Plus Protein Standards (Bio-Rad Laboratories, Hemel Hempstead, UK) were used as protein molecular weight markers. The blotting was performed as previously described (41), following optimization of conditions for all antibodies (summarized in Table 1). Intensities of the bands were measured by densitometry using MCID Basic software (Interfocus Ltd., Cambridge, UK).

3.3. Immunohistochemical analysis

Immunohistochemical analysis was performed on 5µm thick sections of formalin-fixed, paraffin-embedded tumor specimens. Freshly cut sections were mounted on 3-aminopropyl-triethoxysilane (APES)—coated slides, deparaffinized and rehydrated. As the subsequent staining used was peroxidase-based, the sections were first incubated in 0.3% hydrogen peroxide (Sigma-Aldrich Company Ltd, Dorset, UK) for 10 minutes at room temperature to block the endogenous peroxidase activity. The slides were then further protein-blocked with 20% rabbit/goat normal serum in phosphate buffered saline (PBS) with 0.05% Tween 20, for 30 minutes at room temperature. The optimized immunostaining conditions are summarized in Table 2. Incubation with Vector® Nova Red™ chromogen (Vector Laboratories UK, Peterborough, Cambridgeshire, UK) for visualization of immunoreactivity was optimized to 3 minutes for cathepsin S staining and 1 minute for cystatin C staining. The immunoreactivity was visualized by incubation for 3 minutes with Vector® NovaRed. Staining was evaluated by intensity on a scale from 0 to 2+ (0 corresponding to no staining, 1+ representing intermediary staining and 2+ corresponding to most intense staining observed in the positive control used for each staining) and by estimation of positive cells in whole sections of tumors on coded slides. These estimates were made by 3 observers independently and the results were subsequently averaged (inter- and intra-observer error <10%).

3.4. Statistical analysis

Multiple linear regression analysis was used to test whether there is a significant relationship between levels of cathepsin S expression (considering both 26 kDa active form and 37 kDa precursor form) and clinico-pathological and cytogenetical features of the tumor specimens, including size, ciliary body involvement, scleral/extraocular invasion, cell type, presence of PAS-positive loops, chromosomes 3 and 8 status (Table 3). Specifically, a backward stepwise regression model was built to assess the strength of the association of the ratio cathepsin S 26kDa/cathepsin S 37 kDa with the explanatory variables indicated above. A similar analysis was applied to the normalized levels of cystatin C expression and to the ratio of cystatin C/cathepsin S 26 kDa.

4. RESULTS

4.1. Expression levels of cathepsin S and cystatin C in primary uveal melanoma

Cathepsin S levels in uveal melanoma specimens were investigated by immunoblot analysis in 40 primary uveal melanoma specimens (Table 3) detecting both the 37 kDa proform of the enzyme (inactive) and the 26 kDa active form. Cystatin C was detected as a band of approximately 14 kDa. The intensities of the two cathepsin S bands and cystatin C band appeared variable across the panel of specimens analyzed, in conditions of equal loading of cell lysates that was confirmed by the intensity of the GAPDH band (Figure 1A). Furthermore, the bands characteristic for the two cathepsin S forms detected suggested different protein level ratios of the active and procathepsin S across the specimens investigated.

4.2. Statistical analysis of cathepsin S and cystatin C differential expression

Expression of cathepsin S, considering both the sum of pro- and active form of the enzyme and solely the level of active form, as well as expression of cystatin C were subjected to statistical analysis in relation to clinicopathological and cytogenetical characteristics of tumor specimens analyzed (Table 3). Multiple linear regression analysis, based on the backward stepwise

Table 3. Primary uveal melanoma specimens: summary of clinicopathological and cytogenetical features and expression of cathepsin S and cystatin C immunoblotting data

Case Number	Age	Gender	Ciliary body	Scleral/EO invasion	Size (mm/mm) (LBD/height)	Cell type	PAS loops	Cytogenetics 3cen/8cen/8q24	Cathepsin S 26kDa/37kDa	Cystatin C /GAPDH	Cystatin C /Cathepsin S 26kDa
1	74	F	y	-	19.7/7.8	E	+	1/3/3	2.07	n/a	n/a
2	63	M	n	-	9.0/8.0	E	+	2/2/2	0.76	2.90	7.52
3	74	M	n	-	11.4/5.3	E	+	1/2/2	2.13	n/a	n/a
4	75	F	n	-	17.1/7.7	S	-	1/2/4	1.79	0.47	0.53
5	60	F	n	-	12/3.1	E	-	1/2/3	2.81	0.32	0.19
6	64	M	y	-	17.2/10.4	E	+	2/2/2	2.14	n/a	n/a
7	75	F	n	-	10.3/6.7	E	+	1/2/2	3.00	0.55	0.53
8	45	F	n/a	n/a	17.0/8.0	E	+	1/2/3	2.82	0.00	0.00
9	33	M	y	-	17.6/11.7	E	+	2/3/4	0.81	2.93	5.89
10	65	M	n	+	20.3/12.3	E	+	2/3/3	1.50	0.93	0.99
11	53	F	n	-	16.1/12.8	E	-	2/4/4	3.00	0.96	0.81
12	71	M	n	-	13.4/13.1	E	-	2/2/2	1.52	0.87	4.21
13	80	M	y	-	15.0/8.0	E	-	2/2/3	0.00	n/a	n/a
14	51	F	y	-	19.5/5.6	S	-	2/2/2	2.22	0.99	0.64
15	65	M	n	-	14.9/4.6	S	-	1/3/4	2.97	n/a	n/a
16	71	M	y	-	19.5/9.4	E	+	1/2/3	4.42	n/a	n/a
17	70	F	n	-	13.7/9.3	E	+	2/2/2	0.23	n/a	n/a
18	80	M	y	-	18/8.5	E	-	1/3/3	2.67	0.71	1.07
19	68	M	y	-	17.8/11.6	E	-	1/3/3	2.29	1.30	1.32
20	37	M	y	+	18.9/6.8	E	+	1/3/4	3.02	1.65	1.42
21	35	M	n	-	15.0/11.0	S	-	2/2/2	0.04	0.75	16.44
22	52	F	n	-	10.0/11.0	S	-	2/2/2	0.64	0.12	0.89
23	58	M	n	-	8.0/6.0	S	-	2/2/2	0.40	0.39	0.74
24	76	M	n	-	9.0/8.0	S	-	2/2/2	0.11	1.97	15.42
25	63	F	y	-	12.0/10.0	E	-	1/3/3	2.55	0.61	0.80
26	80	M	n	-	15.8/10.3	S	+	1/2/2	1.50	0.48	3.26
27	65	M	y	-	16.0/10.0	E	+	1/3/3	4.92	0.17	0.85
28	64	M	y	-	12.0/7.0	E	+	2/3/5	0.42	1.23	1.83
29	60	F	n	-	12.0/10.0	E	+	2/2/2	0.31	0.62	1.00
30	55	F	n	-	13.0/6.0	S	-	2/2/2	2.22	1.83	3.05
31	46	F	n	-	8.0/3.0	S	-	2/2/3	1.90	0.53	1.34
32	68	F	y	-	14.0/8.0	E	+	1/3/5	2.54	0.66	0.42
33	57	F	y	+	9.0/4.0	E	-	1/3/4	0.63	1.08	1.89
34	65	F	n	-	11.0/7.0	E	-	2/2/2	0.22	1.29	7.94
35	56	M	y	+	8.0/4.0	E	+	2/2/2	0.45	0.52	1.07
36	65	M	y	+	17/6.4	E	+	1/3/3	3.14	0.12	0.27
37	60	F	n	-	14.1/3.2	E	-	1/2/3	3.61	0.25	0.27
38	35	F	y	-	15.6/10.9	E	-	1/3/3	4.13	0.75	0.76
39	65	M	y	-	16.3/8.2	E	-	1/2/2	1.88	n/a	n/a
40	68	M	y	+	20.7/10.2	E	-	2/2/2	1.16	0.28	3.06

Age of patients at treatment is given in full years. Gender: M, male; F, female. Ciliary body involvement: y, yes; n, no; n/a, data not reported. Scleral/extraocular (EO) invasion: +, yes; -, no; n/a, not reported. LBD, largest basal diameter. Cell type: E, epithelioid cells present (epithelioid and mixed type tumors); S, spindle-only type tumors. Periodic acid-Schiff (PAS)-positive loops: +, present; -, absent. Cytogenetical characteristics refer to markers for centromeric regions of chromosome 3 (3cen) and 8 (8cen) and c-myc oncogene at 8q24. Cathepsin S expression is presented as the ratio of active 26 kDa cathepsin S and inactive 37 kDa procathepsin S forms. Cystatin C protein level is normalized to corresponding GAPDH protein level; intensity of the respective bands was measured by densitometry on films resulting from the sequential probing of the blots with anti-cystatin C and anti-GAPDH antibodies; n/a - in 8 cases, due to the size of specimens, cystatin C level could not be evaluated. Ratio cystatin C/cathepsin S 26 kDa represents the ratio of inhibitor to active form of enzyme.

method, revealed that total cathepsin S was not significantly associated with any of the explanatory variables considered. However, the ratio cathepsin S 26kda/cathepsin S 37kDa was significantly associated with the status of chromosome 3 while no significant association was detected for any of the remaining explanatory variables. In particular, tumor specimens with disomy 3 showed significantly smaller values of cathepsin S 26kda/cathepsin S 37kDa than monosomy 3 specimens (test statistics t_{37}=5.6; p=0.001) (Figure 1B).

A similar analysis showed that cystatin C level (expression normalized to GAPDH) was not significantly associated with any of the explanatory variables of the tumor specimens (Table 3). Conversely, the ratio cystatin C/cathepsin S 26kDa, describing the proportion of inhibitor to the active enzyme form, was significantly smaller in monosomy 3 tumors than in disomy 3 tumors (test statistics t_{29}=3.43; p=0.02) (Figure 2). Association of this ratio did not reach statistical significance with the other variables (Table 3). The latter analysis was based on the logarithmic transformation of the variable cystatin C/cathepsin S 26kDa in order to fulfill the assumption of normality that is required in the regression analysis.

4.3. Immunostaining of cathepsin S and cystatin C in primary uveal melanoma tumors

The distribution and characteristics of the immunoreactivity for cathepsin S and cystatin C were evaluated in sections of twenty-four randomly selected, wax-embedded primary uveal melanoma specimens, of which eleven specimens had been included in the immunoblot analysis of the levels of cathepsin S and cystatin C proteins. The immunohistochemical staining made use of the same antibodies employed in the immunoblotting and therefore the immunoreactivity detected was interpreted as the sum of the pro-cathepsin S

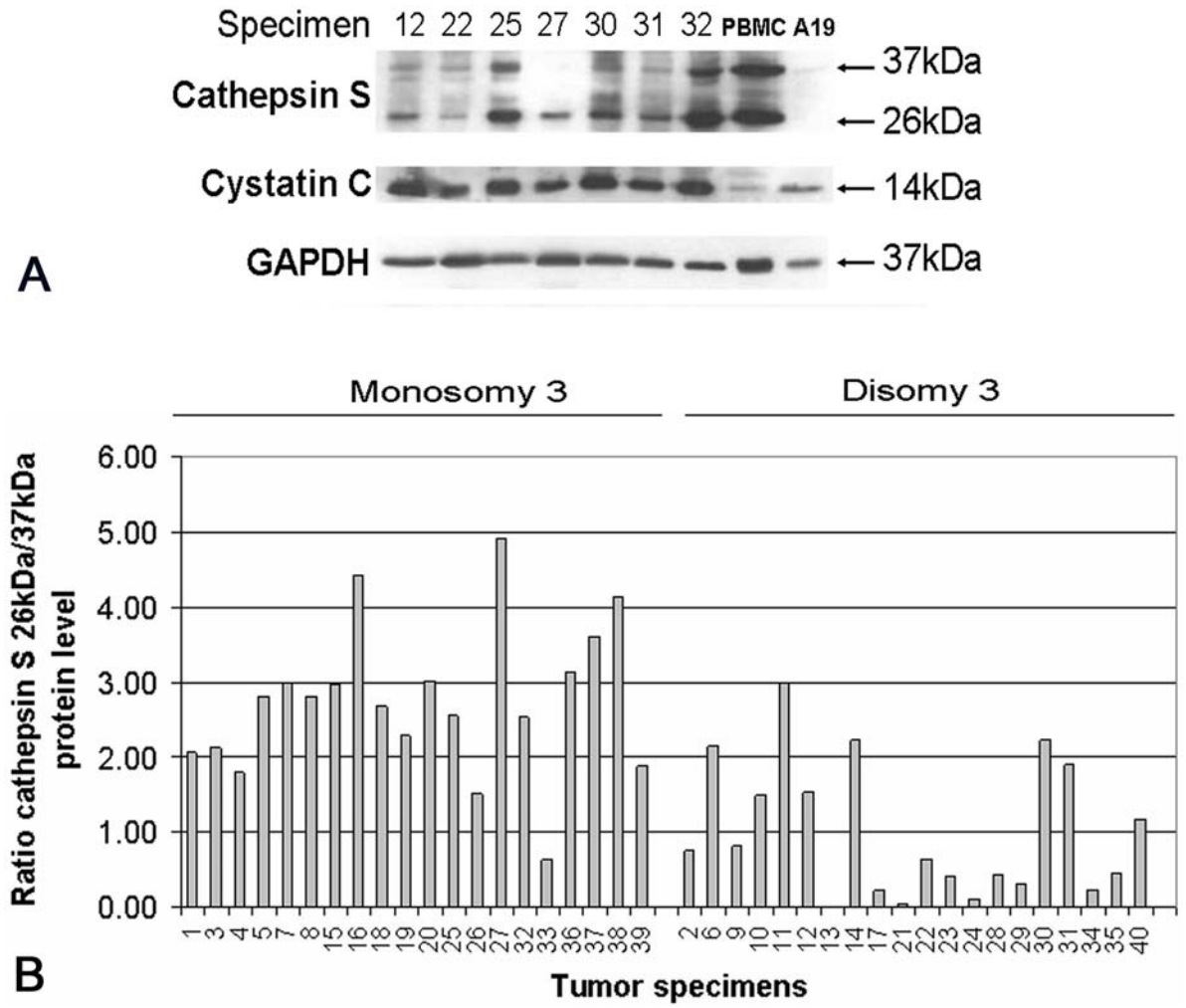

Figure 1. Cathepsin S and cystatin C immunoblot analysis in primary uveal melanoma specimens. (A) Representative Western blot of some tumor homogenates probed sequentially with anti-cathepsin S, anti-cystatin C and GAPDH antibodies. PBMC, peripheral blood mononuclear cells lysate, positive control for cathepsin S; A19, ARPE19 retinal pigment epithelial cells lysate, positive control for cystatin C (55). The anti-cathepsin S antibody recognized both the procathepsin (inactive) form (approximately 37 kDa) and the active mature cathepsin S form (approximately 26 kDa). Cystatin C was identified as a band of about 14 kDa, corresponding to the mature form of the inhibitor. GAPDH, used to confirm equal loading of samples, was identified as a 37 kDa band. (B) Ratio of protein levels of active 26 kDa cathepsin S and inactive 37 kDa procathepsin S in 40 primary uveal melanoma tissue homogenates. Specimen codes correspond to case numbers in Table 3. Intensities of the respective bands were measured by densitometry on films of the same exposure time. Specimens were grouped for graphical representation according to the status of chromosome 3 in the respective tumors, which was significantly associated with the ratio cathepsin S 26kda/cathepsin S 37kDa.

and active cathepsin S and it is referred herein as cathepsin S immunoreactivity.

Cathepsin S immunoreactivity was detected both in epithelioid-type and spindle-type of tumor cells, with the range of cells positive varying between 1% and up to approximately 50% within each tumor. The intensity of staining was also variable, with some cells or areas reaching the intensity (++) similar to that in tonsil tissue used as positive control (Figure 3 A, B) and some cells with appreciably less intense (+) staining (Figure 3 C). It was noted in particular that positivity was often found at the margin or infiltrating edge of the tumor (Figure 3 C, D). In addition, strong positivity (++) was present in the vascular endothelium of the tumor-associated blood vessels, which was also used as an internal positive control (Figure 3 C, D, E).

Cystatin C immunoreactivity was detected in a much lower proportion of tumor cells, while present in the plasma component of blood vessels and adjacent choriocapillaris. Where present in tumor cells, cystatin C staining was diffuse (Figure 4 A) and appeared less

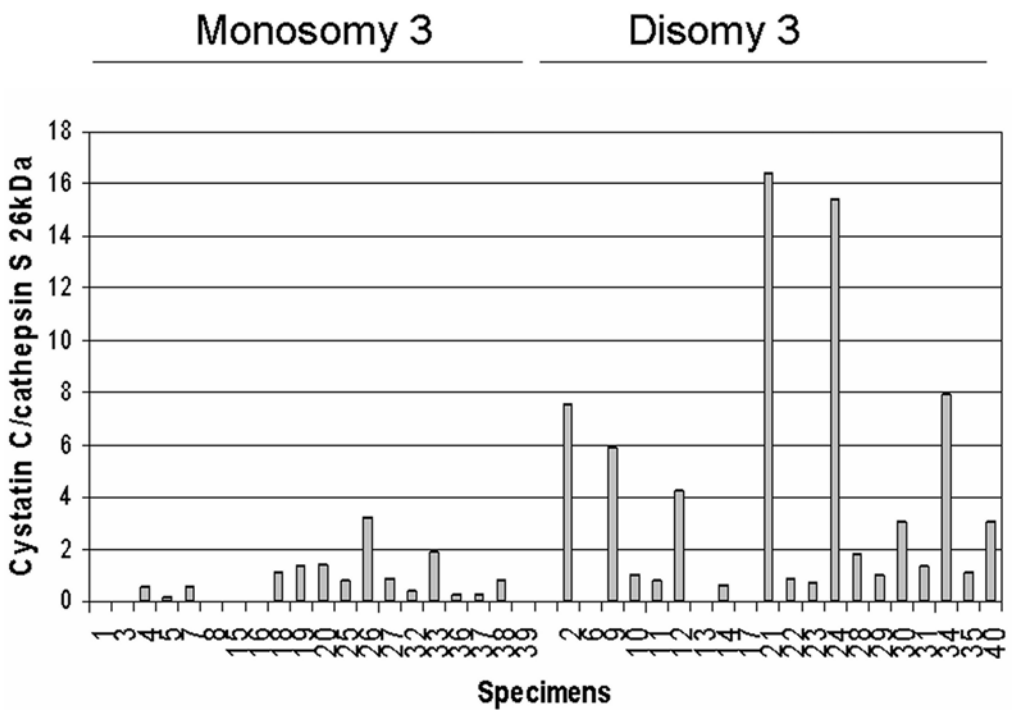

Figure 2. Ratio inhibitor/active enzyme (cystatin C/cathepsin s 26 kDa) is significantly associated with status of chromosome 3 in uveal melanoma specimens analyzed (multiple linear regression analysis; test statistics t_{29}=3.43; p=0.02). Specimen codes correspond to case numbers in Table 3.

abundant than in retinal pigment epithelial (RPE) cells, which were used as internal positive control (Figure 4 B).

5. DISCUSSION

Our investigation revealed a significant relative increase of active cathepsin S in aggressive, highly metastatic monosomy 3 uveal melanoma tumors, as compared with disomy 3 tumors. In addition to unveiling a novel correlation between a specific cysteine protease activity and the strongest predictive factor for metastatic behavior in primary uveal melanoma, the present study pointed to post-translational changes coupled with an imbalance in the endogenous regulatory mechanism as determinant factors for the increase in cathepsin S activity in tumors. Thus, although the overall level of cathepsin S, accounting for pro- and active forms of the enzyme did not increase significantly, the level of active cathepsin S appeared elevated in the aggressive uveal melanoma tumors. In addition, the low and non-changing expression of the endogenous inhibitor cystatin C further contributed to the increased specific cysteine protease activity associated with the aggressive tumors.

The experimental evidence supporting causal roles for cysteine cathepsins in the initiation and progression of cancer is far more limited than that linking matrix metalloproteinases or serine proteinases to cancer. This may be due, at least in part, to a bias towards studying the latter types of proteinases, known to have a predominantly extracellular function, rather than the cysteine cathepsins which were long believed to be functionally active mostly in intracellular vesicles and compartments at low pH. However, evidence has been gradually accumulating for substantiating extracellular functions of cysteine cathepsins, especially in cancer, and for supporting the hypothesis that altered cysteine cathepsin expression and/or altered cystatin C concentration within cell microenvironments have critical consequences in the development and metastasis of cancers (13, 42-44). In this context, the biochemical properties of cathepsin S render it as a unique member of its family being stable and maintaining substantial activity even at neutral pH (33, 34).

The imbalance in expression between various cathepsins, including cathepsin S, and their inhibitor cystatin C was evidenced in other types of cancer and pathological states being mostly, but not exclusively, associated with extracellular matrix breakdown. For example, elevated levels of cathepsins B, H and/or L with a significant reduction in the relative cystatin C to enzyme ratio were characterized in breast (45), ovarian (46), prostate (47) and colon cancer (48). Also, in cardiovascular disease, human atherosclerotic plaques and aortic aneurysmal lesions were shown to have increased lysosomal cysteine protease/cathepsin S content and decreased levels of the endogenous inhibitor cystatin C, suggesting an imbalance that would favor matrix degradation and remodelling in the arterial wall (49-51). A functional interaction between cathepsin S and cystatin C in cancer pathology was strongly and elegantly suggested by a study in a mouse model of pancreatic islet cell

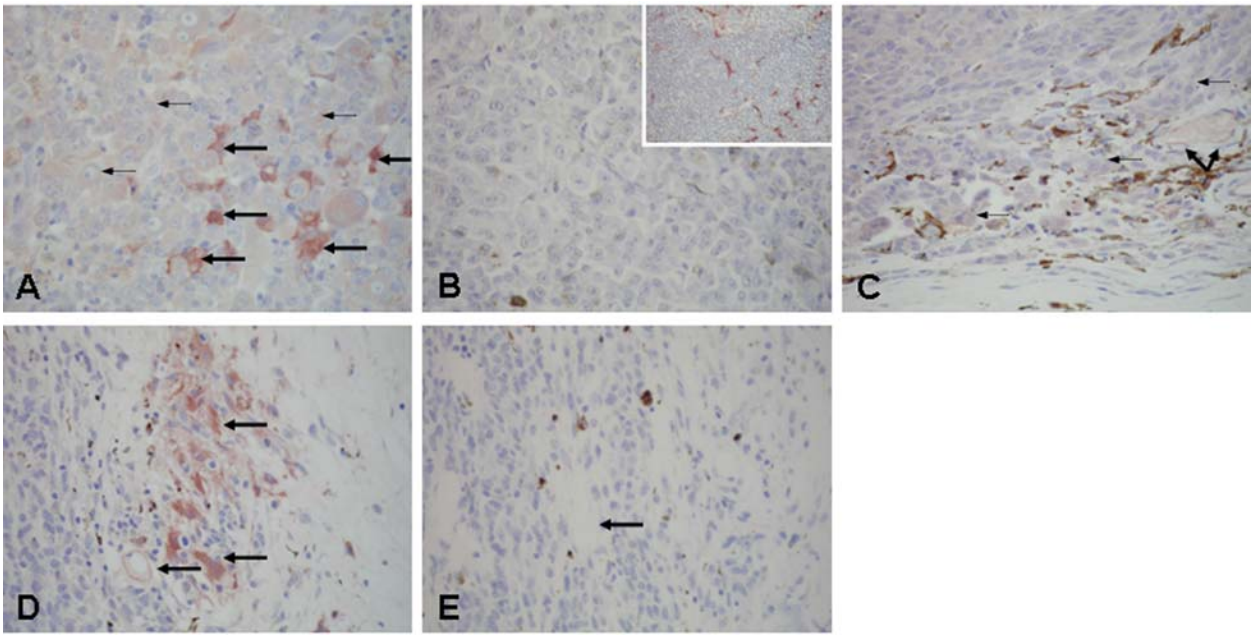

Figure 3. Cathepsin S immunoreactivity in primary uveal melanoma tumors. (A) Monosomy 3 tumor with approximately 50% of cells positive for cathepsin S, with moderate (+; small arrows) and high intensity (++; big arrows) of staining, mostly in epithelioid-type cells, but also in some spindle-type cells (specimen exemplified is #32, Table 3). (B) Negative control of the same specimen as in (A); inset, tonsil tissue used as positive control for staining (++). (C) Disomy 3 tumor (specimen #12) with cathepsin S staining (+) in less than 10% of tumor cells (small arrows), alongside blood vessel endothelium, used an internal positive control (big arrows). (D) Intense cathepsin S staining (++ level) in the cells in the leading margin of the tumor (specimen #40), infiltrating the choroid, similar to the staining of the tumor blood vessel endothelium (arrows). (E) Negative control for staining, same specimen as in (D); note lack of staining of the endothelium.

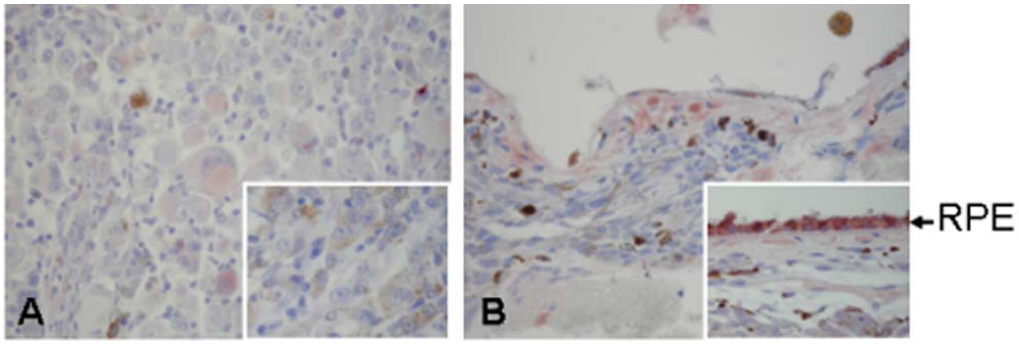

Figure 4. Cystatin C immunoreactivity in primary uveal melanoma specimens. (A) Staining specific for cystatin C was detected at relatively low level in some tumor cells (specimen exemplified is #32); inset, negative control for staining, same specimen. (B) Immunopositivity for cystatin C detected in RPE cells and choriocapillaris, but not tumor cells, next to the leading edge of the tumor (specimen #40); inset, RPE staining above the tumor, used as internal positive control.

carcinogenesis with well-characterized increased level of cathepsin S activity, in which the phenotypes resulting from either selective cathepsin S deficiency or absence of cystatin C were opposite (52). This study however did not simultaneously investigate the relative levels of the enzyme and its inhibitor present in the tumor tissue.

The predominant expression of cathepsin S by cells localized close to the margin of the uveal melanoma tumors strongly suggests a role for this enzyme in the invasive phenotype and malignant behavior of this tumor. Potential substrates include extracellular matrix proteins such as collagen and laminin, cell-adhesion proteins such as cadherins and/or matricellular proteins. However, the increased level of active cathepsin S coupled with a relative downregulation of cystatin C in the tumor overall may translate into other functions. Interestingly, cathepsin S was reported to cleave osteonectin (13), a matricellular protein whose increased expression was previously shown by us to associate with blood vessel-rich uveal melanomas (53). In the light of our present results, the investigation of osteonectin as a substrate for cathepsin S in uveal

melanoma appears very promising and may unveil a pathway linking cathepsin S to induction of angiogenesis in this type of tumor. Cathepsin S was demonstrated to be involved in the induction of angiogenesis associated with pancreatic cancer by degrading anti-angiogenic peptides derived from type IV collagen and generating pro-angiogenic peptides from laminin 5 (52). In addition, the endothelium-derived cathepsin S was shown to have an essential role in the extracellular matrix degradation associated with angiogenesis (28). Consequently, the high level of expression of cathepsin S in the vascular endothelium of the uveal melanoma-associated blood vessels may also contribute to angiogenesis in the tumor. In addition, recent genetic ablation studies in mice attributed a possible role in resistance to apoptosis to cathepsin S, alongside cathepsins B and L (54). Investigation of such a role for cathepsin S in uveal melanoma should be of value since this type of tumor appears to have an intrinsic resistance to apoptosis (41).

In vitro assays of colon carcinoma-related cathepsin B suggested the requirement for an extracellular activation of the enzyme, with a possible implication of the secreted cystatin C in this process (48). Although the possibility of cystatin C involvement in the activation of cathepsin S in uveal melanoma cannot be ruled out, our findings of increased relative level of active cathepsin S in primary uveal melanoma tissue lysates strongly suggests that the activation through post-translational modification(s) of the enzyme occurs intracellularly, most likely before the functional interaction with the secreted cystatin C. The regulation of the activation mechanisms remains to be investigated and may involve inflammatory cytokines which have been shown to augment expression and secretion of active cysteine proteases in other cellular contexts, such as vascular smooth muscle cells and endothelial cells or cultured monocyte-derived macrophages (50).

The pattern of expression of cathepsin S and cystatin C identified by this study suggests that these molecules have specific roles in the proteolytic pathways involved in modulation of the invasive phenotype of uveal melanoma. Further elucidation of how they function in the development and progression of this type of tumor could contribute to the characterization of the proteolytic networks established by cysteine cathepsins and other proteases in cancer thus advancing the anti-proteolytic cancer therapeutic approaches.

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