Expression and prognostic significance of cathepsin L in early cutaneous malignant melanoma

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Cathepsins are papain-like lysosome cystein proteases involved in tumor growth, invasiveness and spread, angiogenesis and alteration in immune and inflammatory responses. We investigated the differences in cathepsin L (CatL) concentrations in primary cutaneous malignant melanoma stage I and normal skin and correlated these values with well-established malignant melanoma prognostic factors.

The study was performed on 36 patients (17 men and 19 women; mean age 54 years; range 21–84 years) with histological confirmed primary malignant melanomas less than 1.5 mm thick. The CatL concentrations were measured in 36 pairs of triton extracts of cytosols prepared from the tumor and adjacent normal tissue samples (matched pairs). The CatL concentrations were determined by commercially available enzyme-liked immunosorbent (ELISA) assay from KRKA (Novo Mesto, Slovenia).

Significantly higher concentrations of CatL were detected in malignant melanomas than in normal surrounding skin (6.73 vs. 1.42 ng/mg total protein (mgp), $p<0.001$). Significant correlations between malignant melanoma and normal skin concentrations for CatL were found. The malignant melanoma CatL concentrations correlated significantly with normal skin ($r=0.38$; $p=0.021$).

CatL concentrations were significantly lower ($p<0.01$) in the malignant melanomas of Breslow thickness ≤0.75 mm, Clark invasion <II, without microscopic ulceration and without vascular invasion (4.14, 4.73, 6.15, 5.29 ng/mgp, respectively) than in the malignant melanomas of Breslow thickness >0.75 mm, Clark invasion of ≥II and ≤III, with microscopic ulceration and with vascular invasion (7.67, 7.41, 9.15, 10.35 ng/mgp, respectively).

Higher CatL concentrations in early primary malignant melanomas indicate its possible involvement in the processes of early metastatic spread and its association with poor prognosis.

Key words: malignant melanoma, cathepsin L, prognosis, tumor marker

The degree of malignancy of malignant melanoma is largely related to its capacity for rapid invasion and metastatic spread, and proteases may play a major role in this process.

Increased level of lysosomal cysteine proteases, cathepsins B, D, H and L have been observed in tissues of primary and metastatic tumors in many cancer types [1–3]. They are involved in degradation of extracellular matrix, thereby facilitating growth, invasion and metastasis, angiogenesis, apoptosis, and in alteration of inflammatory and immune response.

A papain-type lysosomal cysteine protease cathepsin L (CatL) cleaves a wide range of extracellular matrix components (fibronectin, collagen, elastin, and laminin), serum proteins, cytoplasmic proteins and nuclear proteins and has specific implications in epidermal homeostasis and possibly in angiogenesis [4–7]. CatL overexpression, as evidence suggests, is correlated with more aggressive tumor behaviour, early relapse, and reduced ultimate survival in several human solid malignant tumors, namely hepatocellular carcinoma, breast, head and neck, colorectal and lung cancer [8–12].

It has been already immunohistochemically demonstrated that overexpression of CatL takes place in melanoma and nevi, producing a stronger immunohistochemical reaction in the former [13, 14]. The overexpression of CatL in human melanoma cells suggests a functional role of CatL in the early progression of malignant melanoma [15]. Although CatL has previously been correlated with clinico-pathological features

in number of solid malignant tumors [3], information on its prognostic value and correlation with well known pathological prognostic features is limited in relation to early cutaneous malignant melanoma.

The aim of this study was to determine the concentrations of CatL in primary, stage I cutaneous malignant melanomas and normal skin in tumor surroundings. Furthermore, we tried to establish a correlation between CatL concentration and well known pathological prognostic markers e.g. Breslow tumor thickness, Clark level of invasion, ulceration and vascular invasion in stage I cutaneous malignant melanoma.

Patients and methods

Patients. Our study comprised 36 patients (17 men and 19 women; mean age 54 years; range 21–84 years) with histologically confirmed primary cutaneous malignant melanoma. The malignant melanoma was totally excised with a safety edge of 1–2 cm. All lesions were <1.5 mm thick and derived from patients with clinical stage I (T1-2 N0 M0) [16]. The clinical and histopathological characteristics of primary tumors are shown in Table 1.

For the quantification of CatL tissue samples of the size of 2x2x2 mm were excised from the surgical specimen and samples from normal skin at least 2 cm away from the edge of the tumors. They were snap-frozen in liquid nitrogen and stored at –80 °C. The remaining tissue was fixed in 10% formalin and embedded in paraffin for histological examination. Several histological parameters with prognostic relevance were investigated: Breslow thickness, Clark level of invasion, microscopic ulceration on the tumor surface and vascular invasion.

Table 1. Clinical and histopathological characteristics of early cutaneous melanomas

Characteristics	Number
Localization of primary lesion	
Head	4
Trunk	23
Extremities	9
Breslow	
≤0,75	22
>0,76	14
Clark	
0 + I	16
II + III	20
Ulceration	
Yes	9
No	27
Vascular invasion	
Yes	7
No	19
Undetermined	10
Histopathological type	
Lentigo maligna	1
Superficial spreading	32
Nodular	1
Unclassified	2

Tissue extraction and ELISA for CatL. The CatL concentrations were determined in 36 pairs of triton extracts from the tumor and adjacent normal tissue samples (matched pairs). The samples weighing 50 mg were obtained during surgery.

The frozen cut sections were dipped into liquid nitrogen and then pulverized in a microdismembrator (Braun-Melsungen, Melsungen, Germany). For the triton extracts the frozen pulver was dissolved with Tris buffered saline (TBS) (0.02 M Tris-HCl, 0,125 M NaCl, pH 8.5) containing 1% non-ionic detergent Triton X-100 (Sigma, St. Louis, Missouri, USA). The suspension was gently shaken for 3 h at 4 °C.

CatL concentrations were determined by commercially available enzyme-linked immunoassay (sandwich ELISA, from KRKA Novo Mesto, Slovenia). The details of the kits are described elsewhere [14, 17]. The concentrations of the CatL were expressed in ng/mg proteins. Protein content was determined with Bio Rad method.

Statistical analysis. Statistical analysis was performed using the SPSS for Windows program. Differences of CatL concentrations in melanomas and normal skin were analyzed by the Wilcoxon test. Spearman's rank correlations were evaluated for the relations between CatL concentrations in melanomas and normal skin. Differences of CatL concentrations in melanomas and histomorphological variables among various groups of patients were analyzed by the two-tailed t-test and the analysis of variance (ANOVA). The p-values ≤0.05 were considered significant.

The medical Ethics Committee at the Ministry of Health of the Republic of Slovenia approved the study protocol. All the patients included in the study gave their informed consent for voluntary participation in the study.

Results

CatL concentrations in malignant melanomas and normal skin. The mean CatL concentrations in malignant melanomas were significantly higher than in normal skin (6.73 vs. 1.42; $p<0.0001$). Significant correlations were found between malignant melanoma and normal skin concentrations of CatL ($r_s=0.38$; $p=0.021$) (Fig. 1).

Association of malignant melanoma CatL concentrations with relevant prognostic factors. The mean concentration of CatL was compared to Breslow tumor thickness, Clark level of invasion, vascular invasion and ulceration on the malignant melanoma surface (Tab. 2). The mean CatL concentration was significantly ($p<0.01$) lower in malignant melanomas with Breslow <0.75 mm, Clark level of invasion 0+I, in the malignant melanomas without vascular invasion or ulceration.

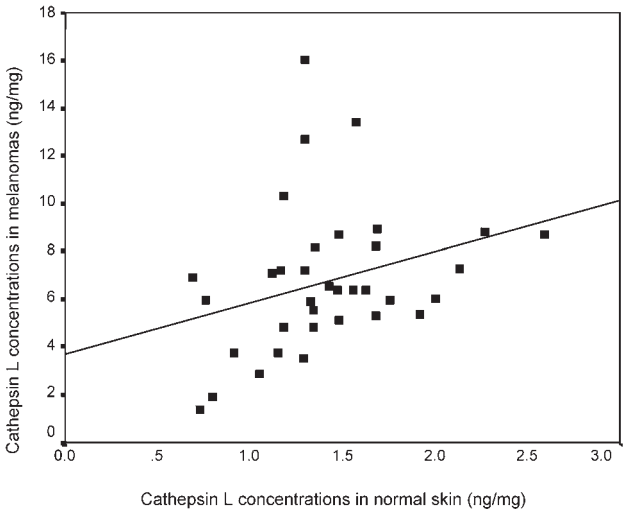

Figure 1. Correlation between melanoma and normal skin cathepsin L concentrations in ng/mg proteins (n=36, r_s=0.38, p<0.021).

Table 2. The mean cathepsin-L concentration in primary cutaneous melanoma within established histopathological prognostic factors

Variable	N	No. of patients	Cathepsin L±SD (ng/mg protein)	p-value
Breslow				
≤ 0.75 mm	22	22	4.14±2.53	0,014
> 0.75 mm	14	14	7.67±2.54	
Clark				
0+I	9	16	4.73±2.21	0,013
II+III	27	20	7.41±2.98	
Ulceration				
Yes	7	9	9.15±4.02	0,014
No	29	27	6.15±2.46	
Vascular invasion				
Yes	2	7	10.35±4.38	0,012
No	15	19	5.29±2.26	
Undetermined	19	10	7.49±2.98	

Discussion

Lysosomal cysteine proteases have been shown to be associated with tumor invasiveness and spread, angiogenesis and alteration in immune and inflammatory responses in many solid malignant tumors including malignant melanoma.

CatL has previously been found in higher concentrations in a number of model tumor systems, both *in vivo* and *in vitro* [19]. Higher CatL concentrations were measured in tumor tissue, indicating its possible involvement in the processes of tumor spread.

In our study, concentrations of CatL differed significantly in early cutaneous malignant melanoma and adjacent normal tissue, the same was true in other studies that dealt with different solid tumors. Furthermore, significant correlations between malignant melanoma and normal skin CatL concentrations were found. To our knowledge, such a finding has not been reported yet.

Only few of published studies have examined the relationship between cysteine proteases and primary early cutaneous malignant melanoma. Furthermore, due to methodological changes over the past 10 years it is difficult to compare contemporary and previous results. In our study CatL concentrations in primary malignant melanoma lesions and adjacent normal tissue were determined with specific ELISA performed on tumor and adjacent tissue extracts [17].

TROY et al [19] demonstrated that CatL concentrations significantly correlate with CatL activity. Cat L activity was higher in tumor than in normal tissue in early, but not in advanced stages of colorectal carcinoma, which could implicate the role of CatL in early colorectal cancer progression. The inverse relationship between CatL activity and stage confirms previous assertions that such activity is important in the early breakdown of barriers to tumor spread from localized to regional and metastatic disease [20]. Furthermore, CatL may be involved in activation of other proteases such as urokinase plasminogen activator involved in later stage of the disease [18].

However, higher intracellular concentration may not imply an increase in proteolytic activity, because this could be well counterparted by enhanced expression of protease inhibitors in certain tumors [17]. It has been widely demonstrated, later on, that CatL does convey some information about tumor progression and that an increase in its secretion by human melanoma cells increases their tumorigenicity [21]. SEVER et al demonstrated that synthetic inhibitors of CatL markedly inhibited murine B16 melanoma cell invasion, which suggests that CatL indeed is a critical factor in tumor growth [22].

However, over the last years possible antagonists of cathepsin action have been exposed. ERVIN et al have recently demonstrated that cystatin C overexpression in B16 melanoma cells reduced their *in vitro* invasion and inhibited haematogeous melanoma metastasis in mice, which results might be ground for targeting cathepsins for anti-metastatic therapy [17, 23].

In general it has been shown that elevated concentrations of CatL in tumor extracts are associated with poor prognosis in a variety of cancer types and does not change significantly with advancing stage [19]. The results of our study show a correlation between CatL concentration and the most important prognostic factors predicting malignant melanoma outcome: Breslow thickness, Clark invasion, tumor ulceration and vascular invasion [24].

More importantly, the concentration of CatL was higher in prognostically worse early melanomas with Breslow thickness >0.75 mm, Clark ≥II ≤III, present ulceration and vascular invasion. Since these prognostic factors are closely related to outcome, one might expect that higher CatL concentrations in primary melanoma would be similarly as-

sociated with poor survival. Analogous to breast, laryngeal and colorectal cancer CatL could become an additional prognostic factor of the progression capacity of primary malignant melanoma next to the other established prognostic factors [20, 25, 26].

In conclusion, our study has shown that the concentrations of CatL differ significantly between tumor and normal skin in patients with early cutaneous malignant melanoma. CatL might also be of prognostic importance in stage I malignant melanoma, but these findings will need confirmation in future studies where the relationship between CatL and patients survival would be investigated.

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