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From Cell Biology to Tissue Engineering

Cathepsin K expression in melanoma is associated with metastases

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Summary. Introduction. Melanoma of the skin shows a tendency to metastasize via lymph or blood secreting matrix metalloproteinases and cathepsins, which enable penetration through the dermis. Cathepsin K acts in cytoplasm of atypical melanocytes and completely cleaves internalized collagen.

Materials and methods. Expression of cathepsin K was analyzed immunohistochemically in 45 melanomas and correlated to morphological and clinical parameters.

Results. During six years follow up, 13 patients developed lymph node metastases and three of them distant metastases. Positive expression of cathepsin K was found in 19 cases. In univariate regression analysis histological type, pagetoid spread, mitotic activity and cathepsin K expression were significantly connected to metastases. Cathepsin K was significantly associated to histologic type, ulceration, pagetoid spread and mitotic rate. In multiple logistic regression adjusted to these variables, cathepsin K was an independent predictor in occurrence of metastases (P=0.015). Median to the occurrence of metastases was 40 months in patients with cathepsin K positive expression and 71 months in patients with cathepsin K negative expression (P<0.001).

Conclusions. In this preliminary study positive expression of cathepsin K in melanoma of the skin is associated with other unfavorable prognostic factors. We consider cathepsin K expression in primary tumor would significantly precipitate occurrence of metastases.

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Introduction

Melanoma represents 4% of malignant skin neoplasms and causes 80% of deaths related to skin cancer. Incidence rate for melanoma has increased by 3-7% in white-skinned population over the past decades (Beddingfield, 2003). Genetic factors known to increase susceptibility to melanoma are skin color and type, number of nevi, occurrence of atypical nevi and positive family history of malignant skin neoplasms, while the most significant environmental factor is exposure to ultraviolet radiation (de Vries et al., 2006). The risk for melanoma in a combination of genetic factors increases several fold (Yang and Cox, 2007). Prognostically significant morphological parameters are growth phase, level of invasion and tumor thickness. Unfavorable factors are the presence of ulceration, high mitotic index, inflammatory infiltration, lymphocapillary invasion and microsatellites (Beddingfield, 2003). Melanoma cells tend to metastasize via lymphogenic and haematogenous route, first degrading extracellular matrix. Malignant melanocytes excrete matrix metalloproteinase which dilute the matrix and cathepsins B, D, H and L which cleave the collagen. The correlation was demonstrated between stronger expression of these proteases and invasiveness and metastatic potential of the melanoma (Hoffman et al., 2003). Cathepsin K is a unique cysteine protease able to completely cleave the collagen helix and plays important roles in bone resorption and homeostasis

of extracellular matrix (Garnero et al., 1998). It is important for the physiological function of organs and depends on matrix degradation and deposition dynamics. Apart of bone, dynamics of extracellular matrix also occurs in the skin (Bossard et al., 1996). Cathepsin K is very low in healthy skin, but proteolytically very active during wound healing and scar formation (Runger et al., 2007). Cathepsin K prevents excessive extracellular matrix deposition, facilitates remodeling and prevents hypertrophic scar formation (Quintanilla-Dieck et al., 2009). Expression of cathepsin K in melanocytes was first described in 2008 (Quintanilla-Dieck et al., 2008). Analyzing the surgical scar near a melanocytic nevus, the authors noticed that some nevus cells were cathepsin K positive. This observation encouraged them to research the role of cathepsin K in melanocytic skin lesions including melanoma. They found strong expression of cathepsin K in melanoma cells which were degrading internalized collagen. Inhibition of cathepsin K increased detection of internalized collagen in lysosomes of melanoma cells. In the next few years, studies about expression of cathepsin K in other types of skin cancer were published. Strong expression of cathepsin K was described in squamous carcinoma (Yan et al., 2011) and basal cell carcinoma (Ishida et al., 2013). In both studies expression was strong in peritumoral fibroblasts and minimal in the carcinoma cells. We would like to analyze cathepsin K expression in malignant melanoma, its correlation to morphological parameters and its correlation in occurrence of metastases.

Materials and methods

The study was performed on biopsies of malignant melanoma of the skin diagnosed in 45 patients from January 1, 2005 to December 1, 2011 at the Clinical Hospital Center Split, Croatia. Data on age, sex and occurrence of metastasis were collected from patients' records. The study included patients who underwent complete removal of the lesion by excision and patients with clinical follow-up. At that period, sentinel lymphadenectomy in melanoma was not routinely performed in the Clinical Hospital Center Split. Disease free survival (DFS) was measured from the date of diagnosis until the date of first occurrence of regional or distant metastasis or the end of the follow-up, in December 1, 2011. Histological samples and paraffin blocks were collected from the archives of Department for pathology, legal medicine and cytology. Original histological slides stained with hematoxylin-eosin were analyzed using Olympus BX51 light microscope (Olympus, Tokyo, Japan). The analyzing parameters were: type of melanoma, invasion according to Clark, tumor thickness according to Breslow, mitotic rate per 1 mm^2, presence of ulceration, pagetoid spread, tumor infiltrating lymphocytes and lymphocapillary invasion. Immuno-histochemistry analysis was performed on formalin-fixed paraffin-embedded histological slides stained with monoclonal antibody anti-cathepsin K (Abcam, Cambridge, Great Britain) diluted at 1/200 and incubated for 17 hours at +4°C in a humidified chamber. After washing, the secondary antibody was applied (Envision Dako, Glostrup, Denmark) and incubated for 30 minutes. After washing with Wash buffer (Dako, Glostrup, Denmark), diaminobenzidine (DAB) chromogen and hydrogen peroxide substrate solution was applied and incubated for 10 minutes. Sections were washed in distilled water, counterstained with hematoxylin and dehydrated in gradient of ethanol, cleared in xylol and mounted using Mounting Medium (Sakura Finetek, Vearlose, Denmark). Staining results were analyzed using Olympus BX51 light microscope (Olympus, Tokyo, Japan). External positive control was peritumoral fibroblasts of basal cell carcinoma. Negative control was the slide in which primary antibody was not applied. Semi-quantitative analysis of cathepsin K expression was performed. Cathepsin K positive expression was defined as fine-grained brownish cytoplasmic immunostaining; according to its density and intensity, expression was estimated as strong or moderate (Fig 1). Data were analyzed using the statistical package MedCalc (MedCalc Software, Mariakerke, Belgium) and the results were deemed statistically significant at P<0,05. Statistical analysis was performed using χ^2test, univariate logistic regression and multivariate adjusted logistic regression analysis and log rank test.

Results

The sample included 45 patients with melanoma of the skin. The median age was 53 years (min-max: 10-77 years). Among them, 25 were males, median age of 54 years (min-max: 22-77 years) and 20 were females, median age of 51.5 years (min-max: 10-75 years). Nine patients had melanoma in situ (Tis), 14 patients had T1 (eleven T1a and three T1b), nine patients T2 (four T2a and five T2b), two patients T3 (T3a and T3b) and eleven patients had T4 (three T4a and eight T4b) (Balch et al., 2009). Positive expression of cathepsin K in atypical melanocytes was found in 19 cases and negative in 26 cases. In the 6 year follow up, 13 patients developed lymph node metastases and three of them distant metastases. We analyzed which histological variables were connected to metastases (Table 1). According to χ^2 test and univariate logistic regression analysis, histological type (χ^2=5.78; P=0.055), anatomic level (χ^2=12.6; P=0.003), depth of invasion (χ^2=7.95; P=0.002), pagetoid spread (χ^2 4.4; P=0.036), mitotic rate per 1 mm^2 (χ^2=7.4; P=0.007), as well as cathepsin K (χ^2=16.3; P<0.001) were connected to metastases.

Cathepsin K expression was compared in different patient groups, according to the analyzed clinical and histological variables (Table 2). There was no significant difference in cathepsin K expression related to sex (χ^2=0.114; P=0.736) or age (Z=1.56; P=0.118). Compared to the group of patients with negative cathepsin K expression, in the group of patients with

positive cathepsin K expression there were more patients with: nodular melanoma than lentigo malignant melanoma and superficial spreading melanoma (χ^2=8.56; P=0.014), invasion of the reticular dermis (χ^2=18.1; P<0.001), tumor thickness over 1 mm (χ^2=18.9; P<0.001) and ulcerated melanoma (χ^2=7.13; P=0.008). In the group of patients without pagetoid spread there were more patients with positive than those with negative cathepsin K expression (χ^2=7.24; P=0.003). Mitotic rate per 1 mm^2 was significantly associated with cathepsin K expression (P=0.001). The presence of tumor infiltrating lymphocytes was not associated with cathepsin K expression (χ^2=0.06; P=0.395).

In further analysis we investigated connection of cathepsin K with some previously analyzed parameters which were significantly connected to metastases. In multiple logistic regression adjusted for histological type, pagetoid spread, mitotic activity and cathepsin K, only cathepsin K was confirmed as an independent predictor in occurrence of metastases [P=0.015, OR (95% CI)=4.2 (1.3-13.3)] (Table 3). Kaplan-Meier survival analysis was performed to estimate disease free survival (the time to evidence of metastasis in lymph nodes or distant organs). Median time to metastasis is 59 months (Fig. 2.). The group of patients with negative cathepsin K expression had almost twice longer median disease-free survival than patients with positive cathepsin K expression, 71 to 40 months (log rank test: 11.21; P<0.001).

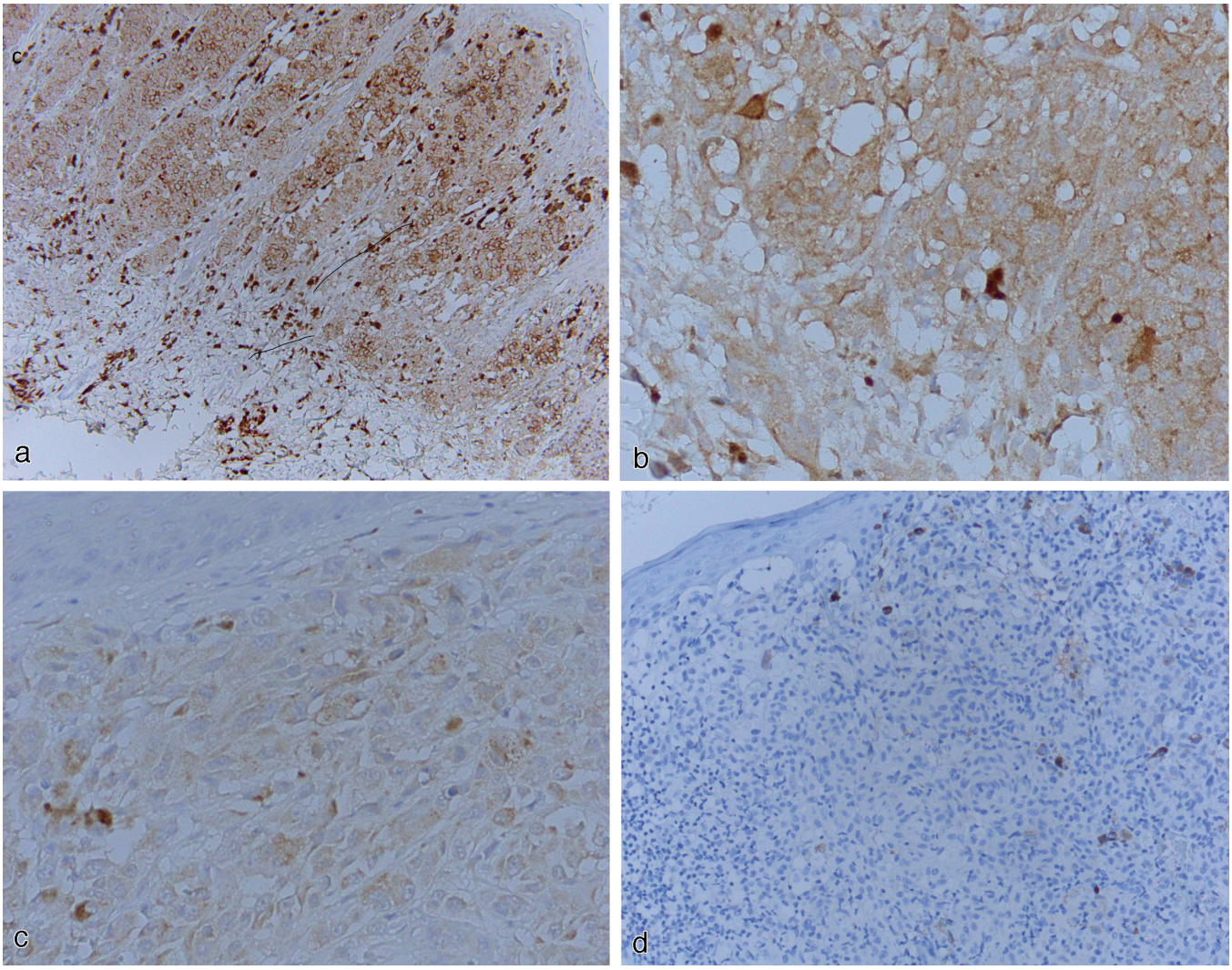

Fig. 1. Immunohistochemical staining of malignant melanoma for cathepsin K shows positive expression in atypical melanocytes **(a)** maniphested as strong **(b)** or moderate **(c)** fine-grained brownish pigment in the cytoplasm. Negative cathepsin K expression in atypical melanocytes surrounded by mononuclear cell inflammation with some melanophages **(d)**. a, x 100; b, c, x 400; d, x 200.

Discussion

Melanoma of the skin displays a tendency to invade local tissues and early metastasize in distant organs via lymph or blood which is associated with unfavorable prognosis. Important steps in the complex process of tumor cell migration, local invasion and metastasizing are degradation of the matrix by powerful proteolytic enzymes (Geller et al., 2009). Proteolytic enzymes such as matrix metalloproteinases and cysteine proteases which degrade extracellular matrix and collagen are particularly responsible for metastatic potential of melanoma cells (Hoffman et al., 2003; Garnero et al., 1998). Important roles of matrix metalloproteinases in tumor invasion and metastasis are demonstrated *in vivo* and *in vitro*, and are associated with tumor stage and prognosis (Nikkola et al., 2005). One study demonstrated expression of powerful lysosomal protease Cathepsin K in melanoma cell lines and melanocytes in skin biopsies (Quintanilla-Dieck et al., 2008). Unlike other matrix metalloproteinases and cathepsins which act extracellulary, cathepsin K acts intracellulary cleaving completely internalized collagen. Cathepsin K inhibitors *in vitro* highly reduce invasion of malignant melanoma cell cultures and the authors assumed its possible important role in tumor cell invasion and metastasis. In our study inspired by findings of Quintanilla-Dieck et al. (2008), expression of cathepsin K in bioptic material of malignant melanoma of the skin is analyzed and compared to morphological parameters (Quintanilla-Dieck et al., 2008). We visualized cathepsin K using indirect immunohistochemistry as fine granular cytoplasmatic positivity in 19 cases. The limitation of our study is inability of standardization of the immunostaining protocol, so interpretation of pathologist is exclusively subjective. The group of patients with negative cathepsin K expression had almost twice longer median time to metastasis occurrence in lymph nodes or distant organs compared to patients with positive cathepsin K expression (log rank test: 11.21; P<0.001).

Of 45 patients included in the study, 25 (55%) were male and 20 (45%) female. In a study about incidence and mortality trends of melanoma in Croatia significantly more males were found (Barbaric and

Table 1. Number (%) of patients according to histological parameters and metastasis.

Parameter	Metastasis		P*	OR (95%CI)	P**
	No (n=32)	Yes (n=13)			
Histological type			0.055	3.5 (1.2-10.5)	0.025
Lentigo maligna	9 (28%)	0			
Superficial spreading	13 (41%)	5 (38%)			
Nodular melanoma	10 (31%)	8 (62%)			
Anatomic level			0.003	3.9 (1.7-8.9)	0.001
Papillary dermis	25 (74%)	2			
Reticular dermis	7 (26%)	11			
Depth of invasion			0.002	4.1 (1.9-9)	<0.001
T0 – T1 ×	17 (53%)	1			
T2 – T4	15 (47%)	12			
Ulceration			0.46		
No	26 (81%)	6 (46%)			
Yes	6 (19%)	7 (54%)			
Pagetoid spread			0.036	4 (1.05-15.9)	0.042
No	9 (28%)	8 (62%)			
Yes	23 (72%)	5 (38%)			
Lymphocapillary invasion			0.954		
No	29 (91%)	11 (85%)			
Yes	3 (9%)	2 (15%)			
Tumor infiltrating lymphocytes			0.239		
No	6 (19%)	5 (38%)			
Sparse	8 (25%)	1 (8%)			
Abundant	18 (56%)	7 (54%)			
Mitotic figures in mm^2			0.007	8.6 (2-37)	0.004
0, 1	27 (84%)	5 (38%)			
>1	5 (16%)	8 (62%)			
Cathepsin K			<0.001	5.6 (2.1-15)	<0.001
Negative	24 (75%)	2 (15%)			
Moderate positive	5 (16%)	3 (23%)			
Strong positive	3 (9%)	8 (62%)			

*: χ^2 test, **: univariate logistic regression, ×: reference level.

Znaor, 2012). Tumor thickness and presence of ulceration are independent predictors of bad prognosis of malignant melanoma; tumor depth according to Clark depends on other histological characteristics, while inflammatory lymphocytic infiltration is a possible prognostic factor (Homsi et al., 2005). We did not confirm correlation between cathepsin K expression and the intensity of lymphocytic infiltration. On the other hand, significant correlation was found between cathepsin K expression and pagetoid spread, but we did not find any similar analysis in the literature to compare. Mitotic index and tumor surface are important prognostic factors of malignant melanoma closely related to tumor thickness and ulceration (Attis and Vollmer, 2007).

In recent research cathepsin K is expressed in primary and metastatic melanomas in different types of tumor cells. Cathepsin K appears to be consistently and strongly expressed in melanocytic lesions and is valuable in distinguishing malignant melanomas from

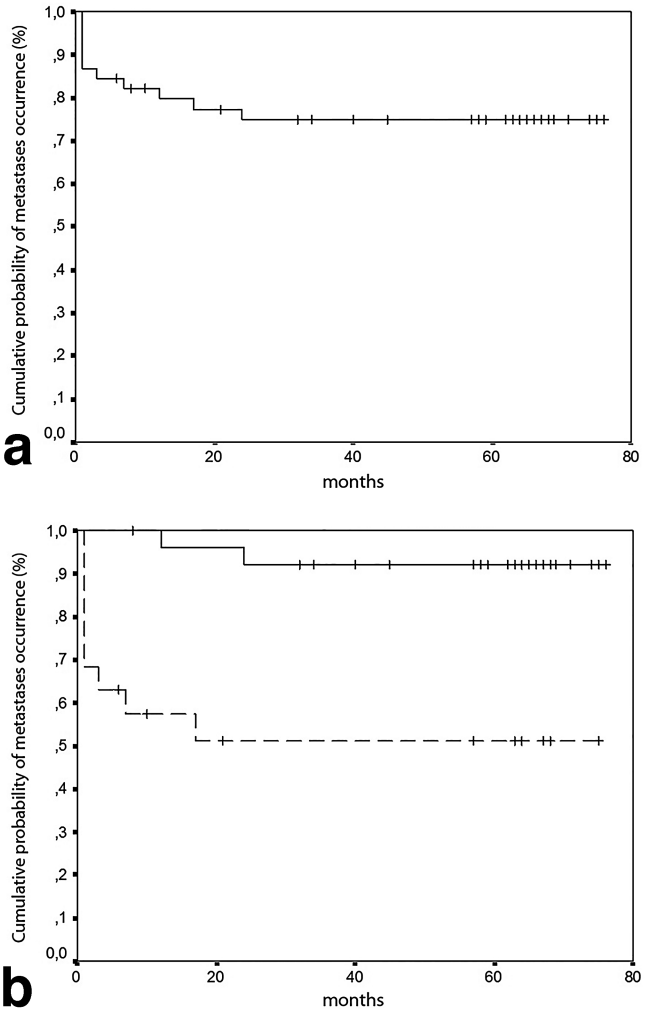

Fig. 2. a. Disease free survival (occurrence of lymph node metastases or distant metastasis) is 59 months (SE: 5 months; 95% CI: 50-68 months). **b.** Patients with negative (full line) and positive (dotted line) expression of Cathepsin K have 71 months (SE: 3 months; 95% CI: 65-78 months) and 40 months (SE: 8 months; 95% CI: 24-57 months), respectively (log rank test: 11.21; P<0.001).

Table 2. Number (%) of patients according to histological parameters and cathepsin K.

Parameter	Cathepsin K		P
	Negative (N=26)	Positive (N=19)	
Histological type			0.014
Lentigo maligna	8 (31%)	1 (5%)	
Superficial spreading	12 (46%)	6 (32%)	
Nodular melanoma	6 (23%)	12 (63%)	
Anatomic level			<0.001
Papillary dermis	23 (88%)	4 (21%)	
Reticular dermis	3 (12%)	15 (79%)	
Primary tumor			<0.001
Tis, T1	21 (81%)	2 (11%)	
T2, T3, T4	5 (19%)	17 (89%)	
Ulceration			0.008
Present	3 (12%)	10 (53%)	
Pagetoid spread			0.003
Yes	21 (81%)	7 (37%)	
Mitotic rate /mm^2			0.001
(median)	0	3.5	
(Q1-Q2)	(0-1)	(1-10.25)	
(min-max)	(0-6)	(1-21)	
Tumor infiltrating lymphocytes			0.394
Brisk	20 (77%)	14 (74%)	

χ^2 test.

Table 3. Multiple logistic regression analysis adjusted for histological type, pagetoid spread, mitotic activity and cathepsin K.

	Metastasis						
	No	Yes	P*	OR (95% CI)	P**	OR (95% CI)	P***
Cathepsin K			<0.001	5.6 (2.1-15)	<0.001	4.2 (1.3-13.3)	0.015
Negative	24 (75%)	2 (15%)					
Moderate positive	5 (16%)	3 (23%)					
Strong positive	3 (9%)	8 (62%)					

*: χ^2 test; **: univariate logistic regression; ***: multiple logistic regression.

the majority of human cancers (Rao et al., 2014).

In conclusion, the expression of cathepsin K in melanoma correlated with known unfavorable prognostic factors and occurrence of lymph node or distant metastases. Our preliminary results should be examined in larger series with known sentinel lymph node status and compared with expression of cathepsin K in melanoma metastases.

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