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CORRELATION BETWEEN PROTEIN nm23 AND CATHEPSIN D WITH CLINICAL AND PATHOLOGICAL FEATURES OF ORAL SQUAMOUS CELL CARCINOMA

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Summary

The most important prognostic factor in oral squamous cell carcinoma (OSCC) is the presence of metastases in regional lymph nodes. Study group comprised of 107 patients with OSCC and control group comprised of 77 specimens of unchanged oral mucosa. The purpose of this study was to determine the immunohistochemical expression of cathepsin D and nm23 protein in OSCC and to compare it to the other clinical and histological features and to the occurrence of regional metastases, in order to assess their prognostic value. In contrast to normal epithelium a diffuse expression of cathepsin D and nm23 protein in OSCC has been found. By comparing the expression of cathepsin D and nm23 protein in tumor cells and in tumor stroma and the type of reaction to the pT, the degree of differentiation, mode of invasion and tumor stroma the following has been found: a significant connection between nm23 protein in tumor cells with the degree of differentiation, nm23 protein with the characteristics of the tumor stroma and mode of invasion and cathepsin D with the degree of tumor differentiation. The results of this research highlight the important role of tumor stroma in metastatic potential of OSCC.

KEY WORDS: *squamous cell carcinoma, nm23 protein, cathepsin D*

POVEZANOST PROTEINA nm23 I KATEPSINA D SA KLINIČKIM I PATOLOŠKIM ZNAČAJKAMA ORALNOG KARCINOMA PLOČASTIH STANICA

Sažetak

Svrha ovog istraživanja bila je imunohistokemijski utvrditi izraženost proteina nm23 i katepsina D u oralnim pločastim karcinomima, usporediti ju s patohistološkim nalazima, pojavom područnih presadnica i procijeniti njihovu vrijednost u prognozi bolesti. Ispitivano je 107 tkivnih uzoraka bolesne i 77 zdrave ustne sluznice. Uočena je pojačana difuzna izraženost proteina nm23 i katepsina D u bolesnom tkivu. Usporedbom proteina nm23 i katepsina D u tumorskim stanicama i tumorskoj stromi, te tipa reakcije s pT, stupnjem diferencijacije, načinom invazije i izraženošću tumorske strome, nađena je znakovita povezanost izraženosti proteina nm23 u tumorskim stanicama sa stupnjem diferencijacije, proteina nm23s izraženošću tumorske strome i načinom invazije, te katepsina D sa stupnjem diferencijacije tumora.Najvažniji prognostički pokazatelj u oralnim pločastim karcinomima su metastaze u područnim limfnim čvorovima. Rezultati istraživanja pokazuju važnost tumorske strome za metastatski potencijal ostnog ustnog pločastog karcinoma.

KLJUČNE RIJEČI: *planocelularni karcinom, protein nm23, katepsin D*

INTRODUCTION

Cathepsins are proteolytic enzymes found and expressed in human tissues which can be divided into three subgroups, depending on their specific proteolytic activity: cysteine protease cathepsines (B,C,H,F,K,L,O,S,V,W), aspartic protease cathepsins (D, E) and serine protease cathepsins (G) (1, 2, 3). Main intracellular physiological functions of cathepsin D are metabolic protein degradation, activation of precursors of bioactive proteins and processing of antigens, polypeptide hormones and growth factors (1, 2, 3). It also plays an important role in regulation of programmed cell death. In the extracellular environment cathepsin D takes part in degradation of extracellular matrix and other processes unrelated to its catalytic activity, such as fibroblast, endothelial and epithelial cell growth induction, neovascularization, tissue remodeling and homeostasis (2, 4, 5).

Some of the cathepsin enzymes, particularly cathepsin D and cystein protease cathepsin B and L, are involved in tumor progression and metastasis (6 - 8, 10, 11). It has been shown that in the majority of breast carcinoma tissue expression of cathepsin D was 2 to 50 times more elevated than in the surrounding fibroblasts or normal glands. According to several clinical studies, the concentration of cathepsin D in the cytosol of breast cancer cells is an independent prognostic factor that correlates with higher incidence of metastasis and shorter survival (2, 6, 9). It has been suggested that some of the cathepsin D increasingly produced by cancerous cells can enter the bloodstream, thus showing elevated concentration of procathepsin-D in blood in patients with metastatic disease (2). Elevated levels of cathepsin D in patients with oral squamous cell carcinoma, oropharynx and hypopharynx carcinoma were associated significantly with the occurrence of regional metastases, regardless of tumor stage and histological grade (10, 11, 12, 13). Cathepsin D is mostly produced in carcinoma cells and stromal macrophages. Originally it was thought that the main role of proteases, such as cathepsin D, in metastasis, is facilitating the invasion of tumor cells by degrading extracellular matrix and components of the basal membrane (7, 5). Later on, it was experimentally confirmed that cathepsin D induces metastatic activity by inducing cell proliferation, more than it increases the invasive potential of the tumor, although the mechanism hasn't been completely elucidated (2).

Discovery of tumor suppressor genes' loss of function as a key element in oncogenesis took further to the assumption that functional losses of other genetic products can, in a similar fashion, allow metastatic spreading. So far around 11 metastasis suppressor genes were discovered, all of which have in common suppression of metastasis dissemination, instead of suppression of tumorigenesis itself (14). Nm23 gene was the first metastasis suppressor genes discovered in 1988, isolated from a melanoma cell culture in mice animal model when it was noticed that cells with a lower nm23 gene expression have greater metastatic potential than those with higher expression. This was later confirmed in other animal and human carcinoma models (15, 16). Low expression of nm23 gene is associated with more frequent occurrence of metastases in breast, cervical and colorectal carcinoma and some other neoplastic lesions (17-20). Researches have also shown that decrease in nm23 expression can be linked to occurrence of tumor metastases in oral squamous cell carcinoma and thus can be considered a prognostic factor (15, 21 - 25). It has also been reported that expression of nm23-H1 protein has a role in invasiveness of the tumor. OSCC cultures with lowest expression of nm23-H1 had invasive morphology, while cells with greater expression had noninvasive, multilayered morphology (26, 27).

Aim

The most important prognostic factor of oral carcinomas is the occurrence of metastases in the regional lymph nodes. Thus, new indicators for biological behavior of tumors and particularly their metastatic potential are being researched and defined, all for purpose of early diagnosis and therapy. Therefore, the aim of this research is to define possible prognostic implications of cathepsin D and protein nm23 expression upon occurrence of regional metastases.

MATERIALS AND METHODS

Research was conducted on the biopsy specimens obtained at the Clinical department of pathology, Clinical hospital Dubrava, Zagreb, Croatia. Biopsy specimens was obtained after clinical diagnosis and surgical removal procedure of the

primary oral squamous cell carcinoma and regional lymph nodes, in the period between January 1st 2000 and December 31st 2004. Study group comprised of 107 patients, 88 men (82.24%), age range 34-81 years (men 57.5 years) and 19 women (17.76%) aged 37-75 years (mean 55 years). Control group comprised of 77 specimens of unchanged oral mucosa of the participants aged 37-81 (mean 58.5 years), out of which there were 58 men (75.3%) and 19 women (24.7%).

Each specimen was immunohistochemically stained with monoclonal antibodies on cathepsin D1 (Novocastra, NCL-CDm) and protein nm23 (Novocastra, NCL-nm23). Primary antibodies were diluted with DAKO REAL antibody dilutent S2022, in ration 1:150 for cathepsin D and 1:200 for protein nm23. Paraffin 5 μm thick sections were deparaffinazed in xylene and rehydrated in a series of alcohol solutions of decreasing alcohol concentration. Presentation of antigen was achieved by heating in the microwave oven at the maximum temperature 100°C for 9,5 and 15 min respectively. Cytrate buffer, pH 6 was used. LSAB method (DAKO, LSAB-system-HRP, K 069 089) and chromogen diaminobenzidine (DAKO, DAB, K3468) were used for elucidation od the reaction. Mayer›s hematoxylin was used for contrast staining.

Gradation of the reactions was defined by the intensity of the reaction and not the percentage of immunoreactive cells. Granulated cytoplasmic reaction was considered positive for cathepsin D, while the positive reaction on protein nm23 was diffused cytoplasmic reaction in more than 10% of cells. Considering the intensity of cytoplasmic staining, reaction could be defined as weak (1) when the intensity was weaker than the one in the positive control, strong (2) when the intensity was comparable to the positive control and very strong (3) when the intensity was stronger than the positive control. Positive control for the expression of cathepsin D and protein nm23 was positive breast carcinoma tissue.

Data were summarized using mean±SD for quantitative variables and number and percentage for qualitative variables. Comparisons between nonparametric quantitative variables were made using Mann–Whitney U-test. Multivariate logistic regression was performed for the occurrence of regional metastases. All statistical analyses were performed with program Statistica for Windows; $P<0.05$ was considered statistically significant.

RESULTS

As seen in the Table 1, differences in expression of both cathepsin D and nm23 between healthy epithelium and OSCC were significant (cathepsin D, P=0.0002 and nm23, P=0.0000).

Types of immunohystochemical reactions of cathepsin D and protein nm23 can be seen in the Table 3. Significant difference in type of reactions between healthy epithelium and OSCC were found for both cathepsin D and protein nm23. Table 3 shows expression intensity of cathepsin D and protein nm23 in tumor stromal tissue. Significant difference was also found for association of expression of nm23 in OSCC cells and differentiation of tumor cells (P=0.0129) (Table 4). Expression of cathepsin D in tumor stroma in association to differentiation of tumor cells was found to be significant (Table 5).

As seen from Table 6, there was significant difference in expression of nm23 protein and type of tumor invasion. Expression of tumor stroma can be associated with expression of nm23 protein (Table 7).

OSCC with metastases in regional lymph nodes have greater and more intense expression

Table 1.

INTENSITY OF EXPRESSION CATHEPSIN D AND nm23 PROTEIN EXPRESSION IN 77 CASES OF HEALTHY EPITHELIUM AND 107 CASES OF SQUAMOUS CELL CARCINOMA

Expression intensity:		Normal epithelium N(%)	Squamous cell carcinoma N(%)	χ^2 test pos. vs. neg.
Cathepsin D	No expression	50(64.9)	39(36.4)	χ^2=13.431 P = 0.0002
	Weak expression	25(32.5)	39(36.4)	
	Strong expression	2(2.6)	27(25.2)	
	Very strong expression	0(0,0)	2(1.9)	
nm23	No expression	25(32.5)	8(7.5)	χ^2=17.340 P = 0.0000
	Weak expression	41(53.2)	35(32.7)	
	Strong expression	8(10.4)	57(53.3)	
	Very strong expression	3(3.9)	7(6.5)	
Total		77	107	

Table 2.

TYPE OF IMMUNOHYSTOCHEMICAL REACTION OF CATHEPSIN D AND nm23 PROTEIN IN 77 CASES OF NORMAL EPITHELIUM AND 107 CASES OF SQUAMOUS CELL CARCINOMA.

Type of expression:		Normal epithelium N(%)	Squamous cell carcinoma N(%)	χ^2 test
Cathepsin D	no reaction	49(63.6)	37(34.6)	χ^2 test= 14.042 P=0.0018
	typical reaction	26(33.8)	37(34.6)	
	Diffuse reaction	2(2.6)	28(26.1)	
	focal reaction	0(0.0)	5(4.7)	
nm23	no reaction	24(31,2)	8(7.5)	χ^2 test= 15.885 P=0.0001
	typical reaction	45(58,4)	78(72.9)	
	Diffuse reaction	8(10,4)	20(18.7)	
	Focal reaction	0(0,0)	1(0.9)	
Total		77	107	

of cathepsin D and nm23 protein in tumor stroma than OSCC without regional metastases (Table 8).

DISCUSSION

Expression of cathepsin D was noticed in only 50% of specimens of the normal epithelium. In OSCC, expression of cathepsin D was significantly higher and of greater intensity than in normal epithelium, which is in accordance with the available literature (2, 28, 29). Expression of cathepsin D in OSCC was more frequent in the basal cell layer, unlike in the normal epithelium in which the expression was more common in basal and parabasal layer. Similar type of reaction in OSCC has been described by Brujan et al., which have observed cathepsin D expression in benign and malignant breast lesions (30). Expression of cathepsin D in stromal tissue has also been described as dependent on the hystological type of tumor. In this research in 51.4% of all OSCC, expression of cathepsin D in stromal fibroblasts was found and was mostly of lower intensity.

In this research no association between expression of cathepsin D and protein nm23 in neoplastic epithelium and tumor size and types of tumor invasion was found. According to the data from the literature, several researches did confirm a correlation between protein nm23 expression and sizing of the tumor, being noted that greater expression was found in tumors of greater size. Thus, the expression of nm23 protein is associated with tumor proliferation and not only with metastasis suppression (15, 17, 18, 27). Expression of cathepsin D has not been associated with tumor size (6).

Table 3.

EXPRESSION INTENSITY OF CATHEPSIN D AND nm23 PROTEIN IN TUMOR STROMAL TISSUE

Expression in stromal tissue		N	%
Cathepsin D	No expression	51	47.7
	Weak	34	31.8
	Strong	21	19.6
	Very strong	1	0.9
nm23	No expression	34	31.8
	Weak	52	48.6
	Strong	21	19.6
	Very strong	0	0

This research has not confirmed the existence of correlation between expression of cathepsin D and protein nm 23 in OSCC with tumor differentiation. Our results show that there is a significantly greater expression of protein nm23 in poorly differentiated carcinoma. In the majority of published papers, lower expression of protein nm23 was found in poorly differentiated, as well as in invasive tumors, even though some researches have not found or supported this correlation (11, 12, 15, 17, 18, 27, 31-36).

Unlike epithelial protein nm23 expression, expression of the same protein in stromal fibroblasts proportionally increases with the share of stromal connective tissue. Expression of protein nm23 and cathepsin D also increased with the tumor size, although this was not significant. Correlation between expression of cathepsin D in tumor stroma and tumor differentiation has been noticed – in moderately and particularly in poorly differentiated tumors, the expression of cathepsin D was greater and of greater intensity than in well differentiated tumors.

Influence of tumor cells on the stromal cells and vice versa has not yet been fully clarified. It is known that cathepsin D produced by stromal cells increases cell migration and acts as a mitogenic

Table 4.

EXPRESSION OF CATHEPSIN D AND nm23 IN EPITHELIUM AND TUMOR DIFFERENTIATION

Expression in the epithelium		Tumor differentiation						Statistical significance
		>75%		25 to 75%		<25%		
		N	%	N	%	N	%	
Cathepsin D	No expression	11	50.0	21	34.4	7	29.2	χ^2 test=4.568 P=0.3344
	Weak expression	8	36.4	20	32.8	11	45.8	
	Strong expression	2	9.1	20	32.8	5	20.8	
	Very strong expression	1	4.5	0	0.0	1	4.2	
nm23	No expression	2	9.1	3	4,9	3	12,5	χ^2test=16.155 P=0.0129
	Weak expression	13	59.1	18	29.5	4	16.7	
	Strong expression	7	31.8	37	60.7	13	54.2	
	Very strong expression	0	0.0	3	4.9	4	16.7	
Total		22	100.0	61	100.0	24	100.0	

Table 5.

EXPRESSION OF CATHEPSIN D AND nm23 IN TUMOR STROMAL TISSUE AND TUMOR DIFFERENTIATION

Expression in stroma		Tumor differentiation						Statstical significance
		>75%		25 do 75%		<25%		
		N	%	N	%	N	%	
Cathepsin D	No expression	18	81.8	30	49.2	11	45.8	χ^2 test=9.613 P=0.0474
	Weak expression	3	13.6	21	34.4	11	45.8	
	Strong and very strong expression	1	4.6	10	13.4	2	8.4	
nm23	No expression	10	45.5	17	27.9	7	29.2	χ^2 test=7.291 P=0.1212
	Weak expression	11	50.0	32	52.5	9	37.5	
	Strong and very strong expression	1	4.6	12	19.7	8	33.3	
Total		22	100.0	61	100.0	24	100.0	

Table 6.

CATHEPSIN D AND nm23 EXPRESSION AND TYPE OF TUMOR INVASION

Expression in stromal tissue		Type of tumor invasion						Statistical significance
		Wide margins		Minor foci		With cords and and single cells		
		N	%	N	%	N	%	
Cathepsin D	No expression	11	55.0	31	59.6	16	45.7	χ^2 test=2.752 P=0.5999
	Weak expression	6	30.0	17	32.7	13	37.1	
	Strong and very strong expression	3	15.0	4	7.7	6	17.1	
nm23	No expression	4	20.0	24	46.2	6	17.1	χ^2test=19.167 P=0.0007
	Weak expression	10	50.0	26	50.0	16	45.7	
	Strong and very strong expression	6	30.0	2	3.8	13	37.2	
Total		20	100.0	52	100.0	35	100.0	

Table 7.

CATHEPSIN D AND nm23 EXPRESSION IN TUMOR STROMA

Expression in tumor stroma		Tumor stroma						Statistical significance
		Poor		Moderate		Rich		
		N	%	N	%	N	%	
Cathepsin D	No expression	22	68.8	20	50.0	17	48.6	
	Weak expression	7	21.9	16	40.0	12	34.3	χ^2=4.438 P=0.3499
	Strong and very strong expression	3	9.4	4	10.0	6	17.1	
nm23	No expression	20	62.5	10	25.0	4	11.4	
	Weak expression	9	28.1	22	55.0	21	60.0	χ^2=21.821 P=0.0002
	Strong and very strong expression	3	9.4	8	20.0	10	28.6	
Total		32	100.0	40	100.0	35	100.0	

Table 8.

CATHEPSIN D AND nm23 EXPRESSION AND OCCURENCE OF REGIONAL METASTASES

N		Metastases-free		Metastases		Statistical significance
		%	N	%		
Cathepsin D stroma	No expression	46	63.0	13	38.2	χ^2=5.987 P=0.0501
	Weak	19	26.0	16	47.1	
	Strong and very strong	8	11.0	5	14.7	
nm23 stroma	No expression	27	37.0	7	20.6	χ^2=6.100 P=0.0474
	Weak	36	49.3	16	47.0	
	Strong and very strong	10	13.7	11	32.4	

factor for tumor cells and fibroblasts by auto and paracrine signaling. Greater expression of nm23 gene also induces expression of cathepsin D. So far, only scarce data on expression of cathepsin D and nm23 gene and particularly their association with clinical and pathohystological characteristics is available and should definitely be investigated further (30). Occurrence of regional metastases and their association with cathepsin D and nm23 protein expression in neoplastic epithelium was not yet established. However, expression of cathepsin D and protein nm23 in tumor stroma was associated with the occurrence of regional metastases. According to our results, OSCC with metastases in regional lymph nodes have greater and more intense expression of cathepsin D and protein nm23 in tumor stroma than OSCC without regional metastases. Overview of the literature shows different data on association between these molecular characteristics and occurrence of regional metastases, even though majority of them observed expression in neoplastic epithelium, while comparable data on expression in tumor stroma are scarce. Expression of cathepsin D in stroma of colorectal carcinoma with greater occurrence of metastases was described. With regard to the expression in tumor cells, greater cathepsin D expression and loss of protein nm23 expression in neoplastic epithelium was associated with the occurrence of regional metastases (6, 10, 15, 17, 18, 32, 36-40). Some authors could not confirm these findings (31, 33, 41 - 43, 45).

CONCLUSION

Greater expression of cathepsin D and protein nm23 in OSCC has been confirmed in comparison to healthy oral epithelium

No correlation between expression of cathepsin D and nm23 protein with the size of the tumor or tumor invasion was found in OSCC. Correlation between expression of protein nm23 in tumor cells and tumor differentiation has been found.

Expression of protein nm23 in tumor stroma has been associated with the share of connective tissue in the tumor stroma. Also, expression of nm23 in tumor stroma has been associated with the type of tumor invasion, while the stromal expression of cathepsin D has been associated with tumor differentiation.

The occurrence of regional metastases has not been confirmed as dependent upon the expression of cathepsin D and protein nm23 in tumor cells.

Significant molecular characteristics, associated with the occurrence of regional metastases of the OSCC are the expression of cathepsin D and protein nm23 in tumor stroma.

Multivariate analysis of all the parameters showed that poor stroma of OSCC, in which cathepsin D is not expressed and which invade with small cohesive streaks, showed lower risk for the occurrence of regional metastases. Moderately-rich stroma of OSCC with abundant inflammation infiltrate and greater expression of cathepsin D have a slightly greater risk for occurrence of regional metastases.

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