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EXPRESSION OF MCSP AND PRAME IN CONJUNCTIVAL MELANOMA.

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ABSTRACT:

Background: To analyse the expression of melanoma chondroitin sulphate proteoglycan (MCSP) and the preferentially expressed antigen of melanoma (PRAME) in conjunctival melanoma (CoM), lymph-node metastases of cutaneous melanoma (LN-met of CM) and conjunctival nevi (CoN) by immunohistology.

Methods: Immunohistology was performed in 70 samples of CoM, 25 of LN-met. of CM and 12 of CoN. and assessed by an immuno-reactive-score (0-12 points). Statistical analysis was performed to disclose relevant differences in the expression-pattern. The diagnostic value of the markers was tested by receiver operating characteristics (ROC-analysis).

Results: MCSP and PRAME were expressed significantly higher in CoM and LN-metastases of CM than in CoN ($p < 0.0001$). Within CoM, a MCSP-expression < 9.0 points meant higher risk for recurrences (Cox HR=3.1) and a shorter recurrence-free survival ($p = 0.002$) than a MCSP-expression > 9.0 points. ROC-analysis showed an area under the curve of 91.3% for MCSP ($p = 0.0002$) and 93.8% for PRAME ($p < 0.0001$).

Conclusions: MCSP and PRAME are differentially expressed in conjunctival melanomas and nevi. MCSP might have an impact on the risk for recurrence in being inversely correlated to the event. Both markers have high potential to discriminate CoM from CoN. Our results indicate that immunohistological characteristics gain relevance in the assessment of conjunctival melanoma.

INTRODUCTION:

In conjunctival melanoma (CoM) most predictable factors are based on clinical or histopathological characteristics [1-6] but little is known about the tumour's biology and protein expression profile.[7] Concerning growth pattern and metastatic pathways, CoM resembles its cutaneous counterpart rather than other ocular melanoma (i.e. uveal).[8-11] The data about the origin of conjunctival melanomas and their rates for local recurrence and metastatic disease show that the tumour's biology needs to be further illuminated in order to understand the underlying mechanisms. New therapeutic strategies are required to reduce recurrence rates and improve survival.[12-14]

Melanoma chondroitin sulphate proteoglycan (MCSP) has become a target in melanoma research when immunotherapy became relevant in cutaneous melanoma. It was identified in the late 1970s as a transmembrane proteoglycan with two components.[15] As a cell surface proteoglycan, MCSP serves as an adhesion receptor influencing cell adhesion and migration.[16] Additionally, it influences cell invasion [17] and proliferation.[18, 19] It has been found that the MCSP-expression correlates to the metastatic potential of a soft tissue sarcoma and melanoma.[20-23] A limited expression of MCSP is shown in normal tissues but is expressed in a high percentage of melanoma lesions.[24]; reviewed by Campoli et al. [25]

The tumour antigen "preferentially expressed antigen of melanoma" (PRAME) is a non-mutated cancer-testis antigen that is expressed in fetal tissue, testis and in cancer, mainly in cutaneous melanoma. PRAME is expressed in 88% of primary cutaneous melanomas and 95% of melanoma metastases.[26, 27] As it is neither expressed in benign cutaneous nevi nor normal skin, it is likely that PRAME expression occurs under melanocyte transformation.[28] It supports cell survival and

suppresses differentiation by interfering with retinoic acid signalling.[29] No data exist about PRAME expression in conjunctival melanoma.

In this study we analysed the expression of MCSP and PRAME in 70 cases of conjunctival melanoma (CoM) by immunohistochemistry and compared the results with lymph node (LN) metastases of cutaneous melanoma (CM) and conjunctival nevi (CoN). The differential diagnosis between conjunctival melanoma and nevi often gives rise to controversy between pathologists or pathologists and clinicians and has an important impact on the therapeutic approach. The established antigens in melanoma diagnostics cannot clarify the diagnosis in all cases. So we examined the potential of MCSP and PRAME to differentiate between these two conjunctival entities. Both antigens have an impact on melanoma progression and may represent therapeutic targets. While MCSP is of special interest as a melanoma specific antigen, PRAME is connected to a very important pathway being a target for modern therapies (RA-signalling pathway). We chose these two antigens to cover these issues for conjunctival melanoma. There does not exist any information about the two antigens in conjunctival melanoma research so far.

MATERIAL AND METHODS:

The study was funded by the Medical Faculty of the University Duisburg-Essen.

Demographic data:

In this study we analysed the clinical data of 70 patients with histologically confirmed malignant melanoma of the conjunctiva (CoM) and performed immunohistology of their tumour samples. The patients were treated between 1974 and 2006 in the Department of Ophthalmology of the University Hospital Essen or Tübingen. The samples were acquired from these departments and the Department of Pathology and Neuropathology of the University Hospital Essen. The mean age was 63.7 ± 16.0 years at the time of diagnosis. The mean follow-up was 69.6 months (min. 0.33, max 317.16 months, median 38.83). Forty cases (57.1%) were presented at the time of first diagnosis and then treated primarily in one of the participating centres. Twenty-two cases (31.4%) had already had a local recurrence at the time of presentation; in 8 cases (11.5%) we had no information about the earlier course of the disease. The tumours were staged following the 6th edition of the TNM-staging-system for conjunctival melanoma (American Joint Committee on Cancer 2002). For demographic- and TNM-data see table 1. Lymph node metastases of cutaneous melanoma (LN-met. of CM) (n=25) and benign conjunctival nevi (CoN) (n=12) served as control groups. Clinical data of these control groups were not available. Primary cutaneous melanoma-samples served as positive controls for MCSP and PRAME. Therefore, we did not use primary cutaneous melanoma as a separate group in this study. We were interested on the expression of the antigens in metastases. Metastases of conjunctival melanoma are numerously rare and not usually excised. So we decided to include lymph node metastases of cutaneous melanoma as control group.

All experiments and procedures were conducted in accordance with the Declaration of Helsinki. An informed consent was obtained from the patients for the analysis of collected samples. The local Ethics Committee of the Medical Faculty of the University Hospital Essen, University of Duisburg-Essen, Germany approved the study.

Immunohistochemistry:

Diagnosis of melanoma was confirmed by hematoxylin-eosin (HE) slides and S-100-, HMB-45- and Melan-A immunostaining. Tissue microarrays (TMA) of conjunctival melanomas (n=70), lymph node metastases of cutaneous melanomas (n=25) and conjunctival nevi (n=12) were assembled with a core-size of 600µm and cut into 5µm thick slices. Suitable donor tissue for the construction of TMAs was identified and marked on HE sections. TMA blocks were constructed with a manual tissue arrayer (Beecher Instruments, Inc, Sun Prairie, WI, USA) with three punch specimens (diameter 0.6 mm) per tumor from corresponding blocks of formalin-fixed and paraffin-embedded tissue. The area of trephination was defined under microscopic control. In small samples 5µm slices were cut and placed directly on object holders. Afterwards, the samples were allowed to dry at 52°Celsius (°C) overnight.

For antigen demasking and deparaffinisation sections were incubated in xylol and rehydrated through graded decreasing concentrations of alcohol (ethanol 100%, 90%, 70%). Afterwards, sections were rinsed with aqueous solution and washing buffer (DCS: WL583CO500). As pre-treatment citrate buffer (pH 6.0) (Zytomed Systems ZUC028-500) was applied to a water bath at 98°C for 20 minutes. Cooling had been allowed for 10 minutes before the samples were transferred to the washing buffer. Afterwards, Peroxidase block was performed for 5 minutes (Dako: S2001), and then the specific antibodies were applied for 30 minutes (PRAME: rabbit, polyclonal, dilution 1:200; MCSP: Mouse IgG₁, LHM2, dilution 1:400, both Biozol

Germany, both dilution-media Zytomed Systems ZUC025-500). A detection system of Zytomed Systems (POLHRP-100) was used. DAB (3,3'-Diaminobenzidin, Zytomed Systems DAB 530) provides brown staining to visualise the complexes. The sections were counterstained with haematoxylin 1:8. Endogenous melanin-pigment was identified in HE-stained samples and was appreciated for the estimation of the immuno-staining. A primary cutaneous melanoma (PRAME+, MCSP+) served as positive control for the TMA investigation. For negative controls specific primary antibodies were replaced by normal serum of the same species. The stained samples were analysed and graded by two independent examiners (HW and FG) using the immuno-reactive score (IRS) (also: Remmele score).[30] Interobserver agreement was assessed by the Kappa-test.[31] In cases of interobserver differences, a third examiner re-estimated the sections and the results were discussed between all three examiners to find a congruent result. The Remmele-score is used to estimate the grade of immunoreaction. The product of the points for staining intensity (no staining: 0; weak staining: 1; moderate: 2; strong: 3) and percentage of stained cells (0%: 0; <10%: 1; 11%-50%: 2; 51%-80%: 3; 81%-100%: 4) results in a score from 0-12.[32, 33]

Statistical analysis

Statistical analysis was performed using Microsoft Excel 2000, SPSS (SPSS for Windows, version 17.0; SPSS Chicago, IL, USA) and StatView for Windows (Abacus Concepts Inc. Version 4.55).

A p-value of $p \leq 0.05$ was considered statistically significant. Results were presented in arithmetic means, standard deviation, medians, ranges, and percentage rates.

Survival analyses to test the relationship of antigen expression, clinical features and recurrences or metastatic disease were performed by the Kaplan-Meier analysis.

Risks were assessed using univariate Cox-regression analysis.

Mann-Whitney-U-test was used for the comparison of antigen expression and nominal variables (2-variables); the Kruskal-Wallis-test was performed for nominal variables with 3 and more variables. Bonferroni-correction was applied to multiple testing with an alpha level set to 0.05. For correlation of continuous variables the Spearman correlation was performed, the results visualised by linear regression.

The unknown cases of the clinical and histopathological parameters were not included in the calculations and were treated as missing values. The cut-off points for MCSP and PRAME of 9.0 points IRS were generated using the mean and median of all cases of CoM (MCSP: mean 8.9; median 9.0 / PRAME mean 9.4).

ROC-analysis (Receiver Operating Characteristic) was performed to analyse the diagnostic value of the antigen expression (sensitivity and specificity) in the distinction of CoM and CoN. In general, the area under the curve is given as an indicator for the accuracy of a tool (here antigen). All points above the diagonal line are better than a random guess. The asymptomatic significance (asymptomatic p-value) of <0.05 indicates that a tool is better than a guess in predicting an event (null-hypothesis: true area=0.5). The cut-off points for each antigen are suggested by the statistical program and they include values from the minimum observed test-value minus 1 to the maximum observed test-value plus 1.

RESULTS:

Demographic data and therapy

Clinical and histopathological characteristics of the conjunctival melanoma-group are summarised in table 1. In our cohort, we could not identify clinical or histopathological risk factors for local recurrence or metastatic disease (data not shown). Analysing therapeutic approaches, 12 patients had only been treated by tumour exzision and had not received adjuvant therapy. Five patients underwent adjuvant Mitomycin C local therapy, 8 cryo coagulation, 12 proton radiotherapy and 18 brachytherapy with ruthenium-106 plaques. Fifteen patients were given other adjuvant therapy. Cox regression analysis revealed an increased risk for local recurrence in patients who got local chemotherapy with Mitomycin C eye drops ($p=0.04$; Hazard ratio (HR) 5.7; 95% confidence interval (CI): 1.1-30.1). In Cox regression analysis, tumours originating from primary acquired melanosis with atypia (PAM) had a 2.4fold increased risk for recurrence compared to *de novo* tumours, (HR for PAM with atypia=1.3 $p=0.58$, 95% CI: 0.52-3.2; HR for *de novo* tumours=0.5; $p=0.46$, 95% CI: 0.11-2.7).

Expression of MCSP and PRAME in CoM, CoN and LN-metastases of CM

The Kappa-test revealed an overall interobserver reproducibility of 64.6%, which is a substantial agreement.[31]

Both antibodies showed a cytoplasmic and nuclear immunoreaction. For MCSP the cytoplasmic staining exceeded nuclear staining. For PRAME nuclear staining was much more intense than cytoplasmic staining. This pattern was observed in conjunctival melanomas, conjunctival nevi, and lymph-node metastases of cutaneous melanoma in equal measure. The highest MCSP-expression with a mean IRS of 11.1 points and a very homogenous expression profile ($SD \pm 1.69$) was found in LN-metastases of CM. CoM expressed MCSP on a high level, as well, but the mean IRS

was significantly lower ($8.9 \text{ points} \pm 2.96$). CoN showed a minor MCSP-expression with a mean IRS of 3.8 points ($SD \pm 2.15$; figure 1, A-C). The difference in MCSP-expression was statistically significant between each of the groups ($p=0.0005$ CoM to LN metastases; $p<0.0001$ CoM to CoN and LN metastases to CoN; figure 2A).

PRAME-expression was highest in LN-metastases of CM (mean IRS 9.84 ± 2.4) and CoM (mean IRS 9.41 ± 3.4). Both groups expressed PRAME on a similar high level (figure 1B). CoN showed a significantly lower PRAME-expression with a mean IRS-score of 2.6 points (IRS score min. 0; max. 6; figure 1, D-F). The difference in PRAME-expression was statistically significant between CoM and CoN ($p<0.0001$) and between LN-metastases of CM and CoN ($p<0.0001$) (figure 2B).

A highly significant correlation was found between the expression of MCSP and PRAME in CoM ($R^2=0.4$, $p<0.0001$).

Expression of MCSP and PRAME in conjunctival melanoma

Statistically, we examined a possible correlation to gender, tumour origin, location of primary tumour (cTNM), pigmentation, infiltration depths (pTNM), predominant cell type, recurrence rate and time of first recurrence, metastatic disease, and occurrence of a second recurrence. We found no statistically significant correlation to these parameters and MCSP expression (data not shown). Tumours with a MCSP-expression <9.0 points IRS had a higher risk for local recurrence than tumours with an IRS-score ≥ 9.0 (Cox-regression analysis, $HR=3.1$; $p=0.003$; 95%-CI: 1.5-6.6; $Wald^2=8.6$). Additionally, tumours with a MCSP-expression <9.0 points IRS had a shorter recurrence-free interval than tumours with a MCSP-expression ≥ 9.0 IRS (Kaplan-Meier analysis; log-rang $p=0.002$; $Chi^2=9.7$) (figure 3). Analysing the subgroups, we found that tumours with a lower MCSP-expression had a higher risk to develop local recurrence and a shorter recurrence-free survival independent from their clinical characteristics (example for epibulbar / nonepibulbar tumours, $p=0.005$,

$\text{Chi}^2=7.8$). The same trend was observed in other characteristics as pigmentation (pigmented/amelanotic; $p=0.002$; $\text{Chi}^2=9.8$), predominant cell type epithelioid ($p=0.02$; $\text{Chi}^2=5.1$), or occurrence of a second recurrence ($p=0.03$; $\text{Chi}^2=4.9$). We also observed a trend that tumours with the origin PAM with atypia (mean IRS 7.9 ± 2.7) had a lower mean MCSP expression than tumours appearing de novo (mean IRS 9.5 ± 2.6). This did not reach statistical significance ($p=0.18$) but gave us a link to a clinical explanation for our findings.

For PRAME, we did not find significant correlations to most parameters (data not shown). In tumours that were confined to the epithelium (pT1, Melanoma in situ), PRAME-expression was significantly lower than in tumours with subepithelial or deeper invasion (pT2-pT4) ($p=0.04$). Tumours classified as pT2, pT3 and pT4 had no significant difference in PRAME expression between the groups. We observed a trend that tumours with an IRS-score for PRAME <9.0 points had a lower risk for metastatic disease (Cox regression, $\text{HR}=0.44$, $p=0.3$; 95% CI: 0.1-2.1) and tended to develop metastases later than tumours with an IRS-score ≥ 9.0 points (log-rang $p=0.3$, $\text{Chi}^2=1.2$). This reached to no statistical significance, possibly due to the limited number of cases with metastatic disease in this cohort.

ROC-Analysis and predictive value of MCSP and PRAME

We performed a ROC-analysis in order to assess the power of MCSP and PRAME to discriminate conjunctival nevi from conjunctival melanoma. For MCSP, the area under the curve was 91.3% (asymptomatic $p\text{-value}=0.0002$; 95% CI: 0.84-0.99, figure 4). For PRAME, the area under the curve was 93.8% (asymptomatic $p\text{-value}<0.0001$; 95% CI: 0.87-1.0; figure 4). These findings make both parameters suitable to differentiate conjunctival nevi from melanoma. Table 2 shows the cut-off points within the IRS score revealing that MCSP and PRAME were diagnostically valuable in the intermediate and high scores (2.5-8.5 pts IRS).

DISCUSSION:

Our results indicate that MCSP and PRAME are differentially expressed in benign conjunctival nevi and malignant conjunctival melanomas. Conjunctival melanoma (CoM) and lymph-node (LN)-metastases of cutaneous melanoma (CM) express both, MCSP and PRAME, at a similar level, whereas conjunctival nevi (CoN) express both antigens on a significantly lower level. Both markers can discriminate conjunctival nevi from melanoma with high significance.

In our cohort MCSP had the surprising effect, that CoM-tumours with lower MCSP-expression had a higher risk for local recurrence and developed their first recurrence earlier than tumours with a high MCSP-expression. Factors that had turned out to be prognostically relevant to the outcome of CoM in clinical studies before did not turn this trend in our study and might therefore be of subordinate predictive relevance compared to immunohistochemical characteristics. But despite our results, this can only be stated as a hypothesis and has to be confirmed by further studies. For explanation, one has to recall the mechanisms associated with MCSP. MCSP is able to influence intracellular signal cascades that have an impact on cell adhesion, motility and invasion.[34] One relevant mechanism in the communication of melanoma cells to extracellular matrix (ECM) is provided by the $\alpha 4 \beta 1$ -integrin and its binding to fibronectin and to the vascular endothelial cell adhesion molecule VCAM [35] which is also expressed in conjunctival melanoma.[36] It is known that the MCSP rat-homologue NG2 collaborates with $\alpha 4 \beta 1$ -integrin for stimulating focal contacts and stress fibre formation in melanoma cells.[22] This may contribute to the metastatic potential of a tumour. On the other hand, it may also be possible, that in cases of a low MCSP expression, the reduced communication of a tumour cell with the ECM and the immune-cells helps to establish a tumour recurrence in the local environment

without being “recognised”. This would explain the trend that a lower MCSP-expression is linked to a higher risk for local recurrence in our cohort of CoM.

Probably, our results could also be explained clinically, as follows: Tumours originating from PAM with atypia had a lower mean MCSP expression and a 2.4fold increased risk for recurrence compared to primary tumours in our cohort. The recurrence-rate may be higher because clinical assessment of PAM with atypia holds the risk of missing a recurrent transformation to melanoma due to their diffuse and variable appearance on the ocular surface. It can be assumed, that the lower mean expression of MCSP in this group leads to our finding that such tumours tend to have a higher risk for recurrence in the whole cohort.

In conjunctival melanoma PRAME is expressed on a high level. Together with the very low expression of PRAME in conjunctival nevi, our results correspond to the reported PRAME-expression in cutaneous melanoma and nevi.[26, 28] Our results indicate that invasive tumours express higher levels of PRAME than intraepithelial tumours. This suggests that PRAME-expression increases under melanoma progression. It is, however, no linear progression but rather seems to divide early (intraepithelial) from advanced (invasive) tumours. PRAME acts by interfering with retinoic-acid—signalling as a dominant inhibitor of the retinoic acid receptor (RAR).[29] Binding of retinoic acid to its receptor initiates the activation of target genes that induce proliferation arrest, differentiation and apoptosis. This is the basis of the antitumoral activity of retinoic acid.[37, 38] Subsequently, the change to, and increase of, PRAME-expression may support tumour progression as the suppression of RAR-signalling promotes tumour-growth and inhibits differentiation. The impact of PRAME on the prognosis of a tumour entity is variable.[39, 40] Our samples showed a trend towards earlier occurrence of metastases in cases with high PRAME-

expression combined with an increased risk for metastases. The statistical value was limited due to the limited number of cases in that subset.

In order to understand the role of PRAME and MCSP in conjunctival melanoma more completely, further genetic studies are required to elucidate the source and mechanism of the increased PRAME- and MCSP-expression in conjunctival melanoma.

MCSP and PRAME have a good diagnostic value to distinguish between conjunctival nevi and conjunctival melanoma. Whether the antigens are able to improve the immunohistological diagnosis in difficult cases has to be evaluated further.

In conclusion, in this study we reported on the expression of melanoma chondroitin sulphate proteoglycan (MCSP) and the preferentially expressed antigen of melanoma (PRAME) in conjunctival melanoma and benign conjunctival nevi. We showed that MCSP and PRAME were differentially expressed in conjunctival melanoma and nevi and can help to differentiate the lesions diagnostically.

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Legends to figures:

Figure 1: Immunohistology of MCSP (A, B, C) and PRAME (D, E, F). Magnification x200. Both Antibodies showed a cytoplasmic and nuclear immunoreaction. MCSP: cytoplasmic > nuclear; PRAME: nuclear >> cytoplasmic. This pattern was observed in conjunctival melanomas, conjunctival nevi, and lymph-node metastases of cutaneous melanoma in equal measure.

Figure 2: A. Boxplot of results of MCSP-expression in the three diagnosis groups. B: Boxplot of results of PRAME expression in the three diagnosis groups.

Statistically significant differences are marked by *. Mean IRS and standard deviation (SD) are given within the box. The horizontal line within the box marks the median. The box itself spans from the 25th percentile (lower end) to the 75th percentile (upper end). The T-lines below and above the box mark the 10th percentile (below) and the 90th percentile (above).

Figure 3: Kaplan-Meier analysis for recurrence-free survival in patients with CoM. The group was divided into MCSP-expression <9.0 points IRS and ≥ 9.0 points IRS.

Figure 4: ROC-analysis of MCSP (continuous line) and PRAME (dotted line). Diagonal reference line for orientation. Area under the curve for MCSP: 91.3 % ($p=0.0002$); for PRAME: 93.8% ($p<0.0001$).

	N=70 CoM (%)		N=70 CoM (%)
Time of presentation	40 first diagnosis (57.1%)	Tumour origin	10 Primary (de novo) (14.3%)
	22 local recurrences (31.4%)		23 PAM with atypia (32.9%)
	8 unknown (11.5%)		16 Nevi (22.9%)
Gender	30 male (42.9%)		10 Recurrence (14.3%)
	40 female (57.1%)		11 Unknown (15.6%)
cTNM	18 T1 (25.7%)	Pigmentation	55 Pigmented (78.6%)
	18 T2 (25.7%)		10 Amelanotic (14.3%)
	17 T3 (24.3%)		5 Unknown (7.1%)
	4 T4 (5.7%)	Recurrence during follow-up	36 Yes (51.4%)
	13 Tx (18.6%)		17 No (24.3%)
pTNM	3 pT1 (4.3%)		17 Unknown (24.3%)
	30 pT2 (42.9%)	Predominant cell type (n=54)	35 Epithelioid (64.8%)
	15 pT3 (21.4%)		12 Spindle cell (22.2%)
	4 pT4 (5.7%)		7 Pleomorph (13.0%)
	18 pTx (25.7%)	Metastases	10 Yes (14.3%)
Location primary tumour	37 Epibulbar (52.9%)		41 No (58.6%)
	21 Nonepibulbar (30.0%)		19 Unknown (27.1%)
	12 Unknown (17.1%)		

Table 1: Demographic and pathological data of the CoM-group with the number of tumours (n=) of the whole group. CoM: conjunctival melanoma.

	≥ pts IRS-cut-off	Sensitivity (%)	Specificity (%)
MCSP Area under curve: 91.3% Asymptomatic significance: p=0.0002
	1.5	98.4	12.5
	2.5	96.9	37.5
	3.5	96.9	62.5
	5.0	85.9	62.5
	7.0	75.0	100.0
	8.5	68.8	100.0

PRAME Area under curve: 93.8% Asymptomatic significance: p<0.0001
	1.5	95.3	50.0
	3.5	92.2	75.0
	5.0	87.5	75.0
	7.0	76.6	100.0
	8.5	73.4	100.0

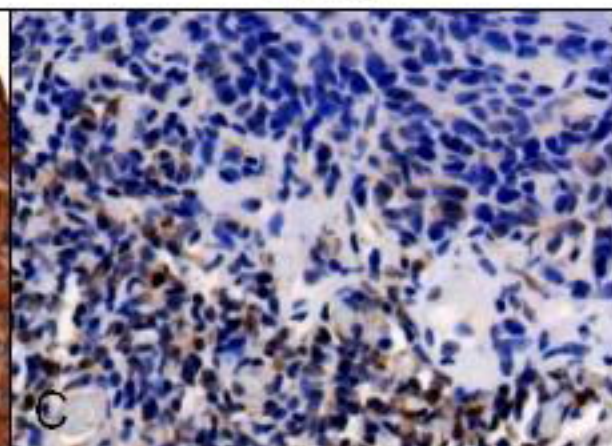
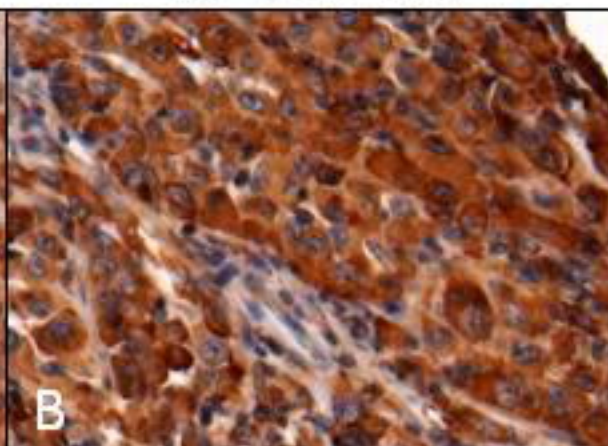
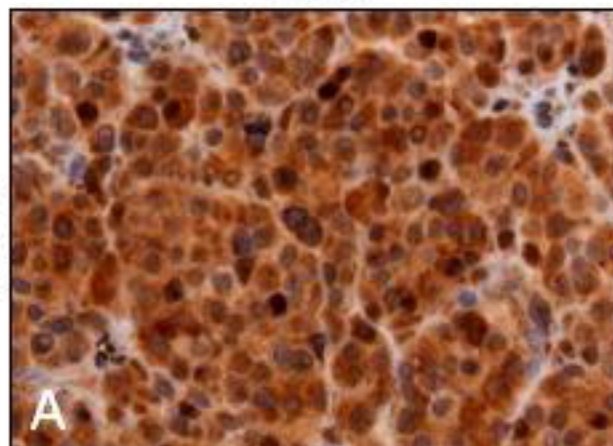
Table 2: Results of the ROC analysis. Presented are parts of the coordinates of the curve to illustrate the possible cut-off values of the IRS of the specific antigens.

CoM

LN-met. CM

CoN

MCSP



PRAME

