Inflammation as a Driver of Vulvar Squamous Cell Carcinoma Progression.

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Research

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Abstract

Background A knowledge on the biology of squamous cell vulvar carcinoma (VSCC) is limited. We aimed to identify protein markers of VSCC tumors stratifying patients by progression risk. Early stage VSCC tumors from patients who progressed (progVSCC) and from those who were disease-free (d-fVSCC) during follow-up, along with normal vulvar tissues were examined by mass spectrometry-based proteomics.

Results In progVSCC vs d-fVSCC tumors, the immune response was the most over-represented Gene Ontology category for the identified DEPs. Interestingly, pathway profiling suggested bacterial infections to be linked to aggressive VSCC phenotypes. Differentially expressed proteins (DEPs) were verified in solid tissues and blood samples of patients with VSCC tumors and vulvar premalignant lesions. High Mobility Group AT-Hook 2 (HMGA2) and Proteinase 3 (PRTN3) were revealed as protein markers predicting VSCC progression.

Conclusions HMGA2 and PRTN3 abundances are associated with aggressive VSCC phenotype, and hold promise as markers for patient stratification. It appears that vulvovaginal microflora disturbances trigger inflammatory response contributing to cancer progression.

Background

Vulvar carcinoma is a rare genital malignancy with age-standardized incidence rate (ASR) of 0.9 per 100 000 worldwide, contributing to the estimated 44 235 new cases and 15 222 VC-related deaths in 2018 [1]. Surgical management is at the cornerstone of treatment for most vulvar cancers, while radiotherapy is added in advanced cases. Patterns of practice in newer strategies, combining surgery, radio- and chemotherapy in advanced and metastatic disease vary [2, 3]. The status of the regional lymph nodes (LN) is the most important prognostic factor for vulvar carcinoma patients, however, it can only be determined with certainty by histological examination of the resected LNs [4], i.e. after surgery with inguinal lymphadenectomy, thus cannot guide surgical decisions.

Ninety percent of vulvar cancers are squamous cell carcinomas (VSCC) which arise *via* high-risk human papilloma virus (hrHPV)-dependent and hrHPV-independent pathways. The first pathway begins with the formation of high grade squamous intraepithelial lesions (HSIL), while the other with a differentiated-type vulvar intraepithelial neoplasia (dVIN) [5]. Although p16-positivity considered a surrogate for hrHPV-positive cases is related with better local control, progression-free, and overall survival [6], so far, there is no difference in therapeutic options for hrHPV-dependent and -independent disease [7]. Clinically relevant, surgery-independent markers are still lacking for VSCC.

The aim of this study was to advance our understanding of the VSCC biology by recognizing biological pathways that drive VSCC progression. For the differential analysis of protein expression, we employed a global mass spectrometry (MS)-based proteomic method, which has so far proved successful in discovery-type experiments in identifying diagnostic cancer biomarkers [8]. Proteomic profiling was

performed to characterize VSCC tumors and in an attempt to identify protein markers that would select tumors more likely to progress.

Results

Identification of Differentially Expressed Proteins Using iTRAQ

We applied the isobaric tags for relative and absolute quantitation (iTRAQ) method, to discover proteins with abundance alterations that are characteristic for the aggressive VSCC tumor phenotype, and to identify the molecular pathways underlying VSCC progression. To obtain a greater depth of protein coverage, 42 samples were analyzed in six 8-plex iTRAQ experiments employing two-dimensional liquid-chromatography (2D-LC), before MS/MS analysis. The specimens included 16 VSCC tumor samples of patients that were found disease-free during the follow-up period (“d-fVSCC” group), 12 VSCC tumor samples obtained from patients who progressed (“progVSCC” group) and 14 normal vulvar tissue samples (controls). MS analysis identified 5509 proteins in this set of samples.

The thresholds for differential expression was set to a protein level fold-change (FC) of ≥ 1.5 and < 0.67 , with a p-value of < 0.05 . With these thresholds, 586 differentially expressed proteins (DEPs) were identified between VSCCs (both d-fVSCC and progVSCC samples) and controls, of which 206 were increased and 380 were decreased in levels in VSCCs (Table S1). DEPs were submitted for GO and KEGG pathway analyses and the results for the top 20 identifiers are provided in Tables S2 and S3, respectively.

We then compared the iTRAQ results obtained for d-fVSCC with those for progVSCC samples at the above-mentioned DEP thresholds and the p-value. The number of DEPs dropped down to 34, of which 27 were increased and 7 were decreased in levels (Table S4). The GO and KEGG pathway analyses were conducted to identify significantly enriched GO functional groups and pathways for the DEPs in d-fVSCC and progVSCC samples. Interestingly, the identified set of significantly enriched GO biological process terms were different from those differentiating VSCCs from controls, and the immune response emerged as the most prevalent (Table S5). KEGG pathway analysis also revealed immune-related pathways among those dysregulated in VSCC (Table S6). For pathway analysis, DEPs with significant alterations in quantity were also submitted to core analysis in Ingenuity Pathway Analysis (IPA). The ten canonical pathways most significantly affected in progVSCC compared to d-fVSCC samples (with a 1.2 threshold for the FC value), presented in Fig. 1, further emphasized the importance of inflammatory reactions that accompany VSCC progression.

Verification by parallel reaction monitoring (PRM)

Global techniques, such as iTRAQ, need to be systematically verified by proteomic experiments with more sensitive, accurate and cost-effective methods of quantifying proteins in larger sample sets, such as the

targeted method of PRM [8]. To verify our DEPs we employed PRM which has lately been shown to be highly specific [9].

Based on the results of the first phase of the project, i.e. the discovery-type global proteomic analysis, a list of 49 candidate protein biomarkers of VSCC progression was generated (Table S7). The levels of these proteins was measured in 14 control, 5 HSIL, 25 d-fVSCC, 26 progVSCC and 25 prosVSCC (primary VSCC from patients enrolled prospectively) samples by PRM analysis using stable-isotope-labelled peptide standards. Twenty-three proteins were found to be significantly differentially expressed between d-fVSCC and progVSCC tumors: 15 were increased and 8 decreased in levels with FC ≥ 1.5 and < 0.8 , respectively. Areas under receiver operating characteristics (ROC) curves (AUC) served to assess the feasibility of individual DEPs in distinguishing between d-fVSCC and progVSCC samples and between VSCC and normal vulvar tissues (Table 1).

Table 1

The list of 23 differentially expressed proteins (DEPs) identified in the iTRAQ experiment and confirmed using PRM analysis.

d-fVSCC vs progVSCC					VSCC vs normal vulvar tissues			
protein	p-value	adjp	FC	AUC	p-value	adjp	FC	AUC
HMGA2	0.0063	0.0824	14.4	0.73	0.0059	0.0095	45.5	0.76
ANO1	0.0068	0.0824	3.37	0.73	0.6177	0.6312	1.80	0.55
PRTN3	0.0117	0.0981	2.75	0.71	<0.0001	<0.0001	13.3	0.92
UBE2C	0.0070	0.0824	2.72	0.73	0.0001	0.0002	8.89	0.87
KRT18	0.0220	0.0981	2.48	0.70	<0.0001	0.0001	8.18	0.91
S100A12	0.0160	0.0981	2.44	0.71	<0.0001	<0.0001	12.0	0.92
PTX3	0.0359	0.1211	2.31	0.68	0.0535	0.0662	2.68	0.68
RUNX1	0.0192	0.0981	2.06	0.70	<0.0001	0.0001	6.73	0.90
PADI2	0.0192	0.0981	1.89	0.70	<0.0001	<0.0001	3.72	0.88
ISG15	0.0273	0.1069	1.88	0.69	<0.0001	<0.0001	35.4	0.94
ABCC10	0.2133	0.3992	1.75	0.61	0.0068	0.0106	5.55	0.75
IFIT3	0.0811	0.1907	1.74	0.65	<0.0001	<0.0001	16.5	0.91
IDO1	0.8737	0.9126	1.68	0.52	0.0001	0.0001	17.4	0.88
PTPMT1	0.2067	0.3992	1.57	0.61	0.0815	0.0957	1.72	0.67
MX1	0.2550	0.4279	1.50	0.60	<0.0001	<0.0001	25.5	0.93
KRTDAP	0.6183	0.7647	0.88	0.54	0.0034	0.0057	0.49	0.77
OGN	0.0670	0.1852	0.81	0.66	<0.0001	<0.0001	0.20	0.91
A2ML1	0.1499	0.3202	0.76	0.63	0.0329	0.0429	0.65	0.70
EDEM2	0.2844	0.4435	0.74	0.59	0.1983	0.2273	1.85	0.62
ABI3BP	0.0230	0.0981	0.71	0.69	<0.0001	<0.0001	0.16	0.92
PRELP	0.0160	0.0981	0.70	0.71	<0.0001	<0.0001	0.26	0.93
CSTA	0.2925	0.4435	0.69	0.59	0.4850	0.5066	1.12	0.57
SPRR3	0.0811	0.1907	0.62	0.65	0.3964	0.4234	2.07	0.58
adjp, adjusted p-value; FC, fold change of differential protein content; AUC, area under receiver operating characteristics (ROC) curves.								

Immunohistochemistry

From the dataset showing the list of 23 candidate biomarkers of VSCC progression verified with PRM analysis, two proteins, namely High Mobility Group AT-Hook 2 (HMGA2) and Proteinase 3 (PRTN3) were analyzed by immunohistochemistry (IHC) in paraffin-embedded tissue specimens obtained from VSCC patients and from those with premalignant vulvar lesions. The samples of premalignant lesions included HSIL which is related to hrHPV infection (predominantly of HPV type 16) and dVIN, the hrHPV-independent precursor of VSCC. HMGA2 and PRTN3 proteins were selected for this analysis based on their abundance difference between d-fVSCC vs progVSCC and between VSCC vs control samples. Additionally, the AUC values indicated relatively high predictive values of both HMGA2 and PRTN3 to identify VSCC patients at risk of progression.

HMGA2 staining was nuclear or both nuclear and cytoplasmic, and confined to neoplastic cells (Fig. 2). In general, HMGA2 staining was weak in premalignant lesions, while it was strong in VSCC tissues. Also, significantly higher relative numbers of HMGA2-positive neoplastic cells were seen in VSCCs than in vulvar intraepithelial lesions (VIN, i.e. both dVIN and HSIL) (Fig. 2D and E). The percentages of HMGA2-positive VSCC cells were not statistically significantly different between hrHPV-positive and -negative primary VSCC samples. Survival curves of the two groups of patients with primary VSCC tumors categorized by the percentage of HMGA2-positive cancer cells are shown in Fig. 2F. The group with a high proportion of HMGA2-positive cells had a significantly shorter time to progression than the group of patients with a low proportion of HMGA2-positive cells in the tumors.

Increase of HMGA2 level may result from an inhibition of let-7 microRNA family members [10]. Therefore, we examined whether the increased HMGA2 content in VSCC tumors was associated with systemic alterations in let-7 levels. qPCR analysis confirmed a decrease of circulating let-7c levels in plasma of patients with VIN and VSCC patients compared to healthy volunteers (Fig. S1).

PRTN3 staining was cytoplasmic and observed in neoplastic cells, macrophages and lymphocytes (Fig. 3A-D). In general, the observed percentage of PRTN3-positive cells was lower among cancer cells than among the infiltrating non-neoplastic cells. Significantly higher relative numbers of PRTN3-positive cells were seen in VSCC than in vulvar intraepithelial lesions (VIN, i.e. dVIN and HSIL) (Fig. 3E-G). Survival curves of the two groups of patients with primary VSCC tumors categorized by the numbers of PRTN3-positive cancer cells, macrophages and lymphocytes are shown in Fig. 3H-J. Patients with higher proportions of PRTN3-positive cancer cells had a significantly shorter time to progression than those with low PRTN3-positive cancer cell percentages in the tumors. The impact of PRTN3-positive macrophage or lymphocyte counts on survival was statistically insignificant.

PRTN3 screening in VSCC patients' blood

PRTN3 is a cell surface protein, playing an important role in myeloid differentiation. It is secreted by neutrophils during their differentiation [11, 12]. PRTN3 belongs to neutrophil serine proteases, enzymes

that were shown to connect innate immunity and coagulation [13]. The complement and coagulation cascades' pathway (hsa04610) was at the top of the KEGG pathways identified in the discovery phase of the study, which compared d-fVSCC and progVSCC samples (Table S6, Fig. S2). Therefore, to determine whether systemic alterations follow the phenomena observed in the analyzed tissues, the presence of PRTN3 was assessed in patient's blood.

We developed a PRM assay to accurately quantitate PRTN3 protein levels using three stable-isotope-labelled peptide standards. Individual plasma samples obtained from 16 VIN patients, 37 VSCC patients and 15 healthy donors were analyzed with the PRM assay. A specific signal was observed for PRTN3 but close to the background noise, and thus it could not be accurately quantified (data not shown). Therefore, the performance of the verified protein was analysed by ELISA in the serum samples collected from 37 VIN, 74 VSCC patients and 44 healthy women. The results of the measurement of PRTN3 as well as of serum anti-PRTN3 antibodies (ANCA, anti-neutrophil cytoplasmic antibodies) are shown in Fig. 4 AB.

The neutrophil protease PRTN3 [13] is a direct modulator of human platelets [14]. Apart from amplifying inflammatory responses, neutrophils exert antimicrobial functions, involving neutrophil phagolysosomes and neutrophil extracellular traps (NETs) that encompass multiple proteases, including PRTN3 [15]. Systemic alterations in response to cancer-related inflammation include an increase in platelet and neutrophil counts and a decline in lymphocyte counts [16]. Therefore, we sought to determine whether these cell counts as well as the neutrophil-to-lymphocyte ratio (NLR) and the platelet-to-lymphocyte ratio (PLR) in VSCC patients were also associated with disease progression, but no significant differences in those parameters between d-fVSCC and progVSCC patients were observed (data not shown).

Subsequently, by the Kaplan Meier survival analysis, we examined a correlation between these protein levels and time to progression (defined as local or nodal recurrence or cancer-related death) in patients with primary VSCC. Survival curves of the two groups of patients categorized by PRTN3 and ANCA serum levels (with the cut-off points of median protein concentrations) are shown in Fig. 4 CD. The group with higher PRTN3 levels had a significantly ($p = 0.0352$, Gehan-Breslow-Wilcoxon test) shorter time to progression (15 months) than the low PRTN3 group (57 months). The survival curves were not significantly different for patients categorized by ANCA serum levels.

The relevance of HPV status of VSCC tumors

The immune response identified at the discovery phase as a key biological process that leads to the progressive state of VSCC tumors could, at least partially, be explained by the biological consequences of HPV infection. In the subsequent workflow of this biomarker study, larger number of patients were enrolled to associate the obtained results with the hrHPV status of the tumors. Unexpectedly, the difference in relative numbers of HMGA2 and PTRN3-positive cells between hrHPV-positive and -negative primary VSCC samples was found to be statistically insignificant (data not shown). We also compared the values of the analyzed hematological parameters (PLT, MPV, NEU, WBC, NEU%, LY, LY%, NLR and PLR) in patients with hrHPV-positive *versus* hrHPV-negative VSCC tumors, and again found no correlation

(data not shown). Similarly, no difference between PRTN3 and ANCA levels in the sera of these patients was observed (data not shown).

This unpredicted lack of association between these results might be explained by the role of bacterial infections in the VSCC disease course. A *Staphylococcus aureus* infection pathway revealed as second among the top 20 KEGG pathways differentiating d-fVSCC and progVSCC sample proteomes (Fig. S3), along with the microbicidal PRTN3 protein revealed to be increased in level during the VSCC course, suggest that bacterial infections may be linked to aggressive VSCC phenotypes. Thus, we retrospectively reviewed the results of bacterial infection testing of vulvar swabs taken pre- and postoperatively from VSCC patients that were treated at the Maria Skłodowska-Curie National Research Institute of Oncology. Apart from *Staphylococcus aureus*, the reported Gram-positive bacteria detected in the swabs included *Anaerococcus prevotii*, *Clostridium perfringens*, *Collinsella aerofaciens*, *Corynebacterium amycolatum*, *Enterococcus faecalis*, *Fingoldia magna*, *Lactobacillus* spp., *Peptoniphilus asaccharolyticus*, *Peptoniphilus harei* and *Peptococcus anaerobius*. Among Gram-negative bacteria, *Bacteroides fragilis*, *Escherichia coli*, *Fusobacterium nucleatum*, *Morganella morganii*, *Streptococcus* spp., *Prevotella* spp., *Proteus mirabilis* and *Pseudomonas aeruginosa* species were identified. However, as the vulvar swabs are not routinely used for bacterial testing in VSCC patients' management, we were not able to assess the influence of bacterial flora on the disease course. The microbiological examination of the swabs was performed mainly in patients with advanced disease, often characterized by macroscopic necrotic changes and a clinical picture indicating possible infection or inflammation, seldom in patients with early stage disease. Therefore, we performed PCR genotyping of 13 microorganisms known to contribute to vaginal dysbiosis on DNA isolated from 36 primary VSCC tumor samples. A pan-bacterial 16S rRNA gene (data not shown) DNA was found in all but one sample that eventually was excluded from the analysis due to poor DNA quality. The specific bacterial species (out of 11 bacterial species analyzed) were detected in 74% of samples (26/35) (Fig. 5). The most common was *E. coli* (17/35), followed by *B. fragilis* (8/35), *P. bivia* (8/35), *S. agalactiae* (8/35), *S. aureus* (7/35), *E. faecalis* (4/35), *L. iners* (3/35) and *L. gasseri* (2/35). No difference was observed in terms of the identified bacterial frequency distribution in d-fVSCC and progVSCC nor in hrHPV-positive and negative samples.

Discussion

Current knowledge on the molecular biology of VSCC is scarce. In recent years, novel treatments that could exploit the virology of HPV and/or enhance the host immune response have been proposed, still there is a paucity of clinical trials in VSCC [7]. Moreover, individualization of the currently available treatment modalities is hampered by the lack of reliable prognostic factors approved for clinical practice. The FIGO staging was shown not to be fully correlated with VSCC patients' prognosis [17]. Even the tumor-free pathological resection margin distance of < 8 mm, long considered as a significant risk factor of vulvar recurrence, has lately been challenged as being associated with local recurrence [18]. Patients with multiple positive LNs benefit from adjuvant radiation therapy following surgery, yet in patients with a single positive LN the survival benefit still remains debatable [19–21]. Likewise, the role of adjuvant

chemoradiotherapy in patients with LN involvement, as well as of neoadjuvant treatment modalities in advanced VSCC cases are not well defined.

The experimental path we applied, from global proteomic analysis to the targeted and quantitative PRM analysis of specific proteins, is the progress towards understanding the mechanisms underlying VSCC progression. A similar approach was previously confirmed by Sandberg *et al.* [22] while detecting protein alterations related to HPV infection in a set of VSCC tumors. An increasing evidence suggests that HPV-harboring VSCC tumors are less aggressive, though some ambiguities remain regarding the prognostic value of HPV status because of the different HPV testing methods applied, and the low numbers of cases examined in some studies [23–25]. In our study, we analyzed a cohort of early-stage VSCC patients with a long-term follow-up after surgery, which enabled stratification of cases according to treatment outcomes. Tumor proteome pathway fingerprinting with Gene Ontology and Kyoto Encyclopedia of Genes and Genomes was performed to investigate the phenotypic changes driving VSCC tumor growth and promoting its aggressive behavior. We identified the inflammatory response as the most prevalent constituent of the top pathways revealed to be altered based on enrichment or depletion of DEPs in tumors of VSCC patients who progressed, *vs* those of patients who were disease-free over a long follow-up time. This notion, based on VSCC tumor proteomics data combining cancerous and micro-environmental cellular alterations, seems to be particularly justified given no immune-related pathways relevant to DEPs differentiated VSCC and normal vulvar tissues.

The immune infiltrate of VSCC and its precursor lesions by innate and adaptive immune cells, recently reviewed by Abdulrahman *et al.* [26], point towards immunotherapeutic options to augment the current treatment modalities. The results of our “modelling” of VSCC tumorigenesis at the level of regulatory pathways and sets of molecules, suggest that the innate immune response is a key attribute accompanying vulvar cancer progression. Local chronic inflammation and systemic inflammatory reactions that are implicated in cancer development include the activation of platelets and the coagulation system [27]. Local inflammatory reactions may result from a disturbed balance between the host and its commensal microbes and may promote cancer formation [28]. Neutrophil elastase (ELANE), PRTN3 and cathepsin G (CTSG) are the main neutrophil serine proteases (NSPs) released by activated neutrophils at the sites of inflammation, and are mediators of innate immune responses to microbial threats [13, 29]. In response to bacterial infection, in otherwise healthy individuals, NSPs connect innate immunity and coagulation by promoting local thrombus formation and microvascular occlusion, to isolate the pathogens and prevent the systemic spread of invading microorganisms [30, 31]. PRTN3, which – along with CTSG and ELANE – is a potent platelet agonist, induces platelet activation [32, 33].

In our study, the *Complement and coagulation cascades* and *Staphylococcus aureus infection* KEGG pathways were the two pathways most significantly differentiating d-fVSCC and progVSCC samples. Noteworthy, these findings refer to the causes of VSCC progression (not the effects of disease progression) as just the primary tumors were examined in the discovery phase of the study. Commensal *S. aureus* colonization may occur on human skin and in the nose, and up to 40% of adults are reported to be carriers [34]. Bacteria and fungi residing in the female genital tract cause common and recurrent

vulvovaginal microflora disturbances leading to i.a. vulvovaginal inflammation. In otherwise healthy women who contract genital tract infections, *Staphylococcus aureus* is an infrequent but important pathogen [35, 36]. *S. aureus* DNA was demonstrated to be strongly associated with squamous cell carcinoma of the skin [34]. Olejek *et al.* [37] found 90% of VSCC, as assessed prior to surgery, to contain pathogenic bacterial flora, including *S. aureus* species in 16% (7/43) of the analyzed cases. It must be noted, however, that the *Staphylococcus aureus* infection KEGG pathway may not be species-specific. However, there are no data on the vulvovaginal microbiota make-up in cancer patients to support the concept of promoting VSCC progression by specific microbes. Several microorganisms of the vulvovaginal region that cause bacterial vaginosis and trichomoniasis had previously been associated also with cervical cancer [38, 39]. Vaginal dysbiosis is suggested to be a risk factor for HPV infection and persistence. Moreover, there is a trend toward an increased risk of cervical cancer progression (though not reaching statistical significance) in patients with vaginal dysbiosis [38]. Our preliminary PCR genotyping findings do not support an association between the presence of bacterial DNA in VSCC tumors neither with hrHPV infection nor VSCC progression. These results require verification with more throughput (covering more microbial species) and a quantitative method, preferably based on vulvar swabs rather than vulvar tissue samples. Yet, our data clearly confirm the presence of pathogens, including *S. aureus*, within vulvar tumors.

Tumor-educated platelets (TEPs) induce the local and systemic responses to tumors growth and thus a TEP-based liquid biopsy was proposed for cancer diagnostics [40]. In VSCC, the preoperative NLR and PLR values were shown to be associated with the most important prognostic factor, the nodal status [16]. As NLR and PLR counts did not differ between d-fVSCC and progVSCC patients in our study, the results of GO analysis, i.e. identification of *platelet degranulation* GO term in d-fVSCC *vs* progVSCC global proteomic analysis, as well as elevation of serum PRTN3 concentration during VSCC progression and metastasis formation, suggest neutrophil and platelet activation during the disease course, rather than their increased numbers.

In our study, a global proteomic approach revealed a multitude of DEPs in d-fVSCC *versus* progVSCC tumors. The panel of proteins that passed the discovery phase based on their statistically significant differential expression, was verified by PRM analysis. The results of verification with targeted proteomics, by accurately quantitating protein levels in tumors samples, provided a list of reliable candidate biomarkers having “progression-classifier” properties, and these proteins need to be validated in future studies. In the consecutive steps of global data verification and narrowing-down of the list of DEPs, in order to determine the histopathological context and cell localization of the two proteins, we performed IHC analysis of VSCC tumors with anti-HMGA2 and anti-PRTN3 antibodies.

HMGA2 belongs to the family of high-mobility group proteins, the second most abundant chromatin proteins after histones [41]. HMGA proteins are indirect transcription regulators that alter chromatin structure and affect, either positively or negatively, the transcription of a variety of target genes including inducible genes related to the immune system functions [42, 43]. HMGA2 is highly expressed during embryogenesis and rarely in adult tissues [44]. Observed in many solid tumors types, HMGA2

overexpression correlates with poor survival [45]. In tumors of epithelial origin, HMGA2 was shown to be essential for tumor progression and metastasis by inducing the epithelial-mesenchymal transition (EMT) *via* interaction with the TGF β signaling pathway [46]. HMGA2 overexpression, caused by the translocations at the 12q15 region, was first identified in benign mesenchymal tumors [47]. HMGA2 overexpression can also result from down-regulation of let-7 microRNAs [10]. In our study, we have observed decreasing levels of circulating let-7c in plasma samples of VIN and VSCC patients. Recently, HMGA2 was demonstrated by RT-PCR and IHC to be expressed in VSCC tumors but not in the normal vulva tissue [48]. In our study, HMGA2 tissue abundance was associated with the aggressive phenotype of VSCC. HMGA2 is known as a nuclear protein, however, in our VSCC sample set HMGA2 staining was also clearly cytoplasmic. The possibility of the presence of the HMGA2 protein at more than one, just nuclear, cellular compartment was indicated previously, in the approach focused on identifying low-molecular-mass proteins, revealing HMGA2 to be secreted from oral cavity squamous cell carcinoma cell lines [49].

The second verified candidate protein biomarker, PRTN3 (myeloblastin) belongs to a family of NSPs which digest matrix components in inflammation and, independently of their proteolytic activity, possess microbicidal properties [11]. PRTN3 contributes to neutrophil transendothelial migration *via* interaction with CD177 [50]. The PRTN3 protein, derived from mature neutrophils, is detectable in the healthy donors' circulation [51]. The presence of anti-neutrophil cytoplasmic antibodies (ANCAs), predominantly directed against the neutrophil serine proteinase (PR)-3, but also against myeloperoxidase (MPO), is characteristic for granulomatosis with polyangiitis and vasculitis (GPA) [52]. GPA belongs to ANCA-associated vasculitis (AAV), a group of vasculitides characterized by neutrophil-rich inflammation of small vessels and the presence of circulating ANCAs. Interestingly, AAV - including GPA - has been associated with chronic nasal *S. aureus* carriage, which is a risk factor for disease relapse [53]. Bacterial infections lead to neutrophil activation, which triggers NETosis, and indeed in AAV patients NETs were shown to be present in skin lesions, kidney glomeruli and in thrombi [54]. NETs consist of i.a. pro-inflammatory proteins, such as high-mobility group box 1 (HMGB1) that was shown to be elevated in AAV patients' sera [55]. In the pathogenesis of GPA, besides neutrophils, the key effector cells (<https://clinicaltrials.gov/ct2/show/NCT01862068>), also activated platelets are proposed to play an important role [56]. NETs can activate the alternative complement pathway and contribute to thrombus formation while in turn platelets induce NETosis with HMGB1 expression on the platelets mediating the process [54].

In our study, PRTN3 was quantitated along with ANCA in serum samples of VIN and VSCC patients with an intention to identify a secreted protein that might prove to become a circulating biomarker of VSCC progression. Indeed, circulating PRTN3 and ANCA, were found to be elevated in VSCC patients as compared to healthy female donors. This observation seems to be related to neutrophil or platelet activation rather than a change in their numbers. Both local and systemic abundance of PRTN3 found in VSCC patients, suggests similarities in the mechanisms underlying VSCC and GPA development. These analogies might include a key role of bacterial infection in these conditions.

PRTN3 has been proposed as a therapeutic target for example in chronic obstructive pulmonary disease (COPD), an inflammatory condition associated with neutrophilic inflammation [57]. Sivelestat, a selective ELANE and PRTN3 inhibitor (<https://pharos.nih.gov/idg/ligands/sivelestat>), was analyzed in the four clinical trials on lung injury and respiratory failure (ClinicalTrials.gov). Sivelestat was shown to improve the mortality rates of patients with sepsis associated with acute respiratory distress syndrome and disseminated intravascular coagulation [58]. ELANE and PRTN3 along with CTSG are NSPs stored in neutrophil granules and externalized during neutrophil activation at inflammatory sites [29]. In pathological conditions, these NSPs may interact with the complement pathway by cleaving C5a receptor (C5aR/CD88) expressed on neutrophils thereby inactivating its C5a-induced signaling ability, and causing inefficient bacterial clearance [31]. ELANE activity is associated with poor outcomes in numerous malignancies, and a recent study of Lerman *et al.* [59] points towards ELANE as a biomarker and therapeutic target in prostate cancer. ELANE, PRTN3 and CTSG play a regulatory role in noninfectious inflammatory diseases and should be of interest as potential therapeutic targets in cancer management. Recently, Kam *et al.* [60] reported an inhibition of HMGB1 with sivelestat to be able to promote myelodysplastic syndrome (MDS) cell death and alter innate immune responses *via* suppression of NFkB pathways. As HMGA2, another high-mobility group protein, also promotes the release of pro-inflammatory cytokines through regulation of the NFkB pathway [61], one might hypothesize that sivelestat could also block HMGA2, providing an additive benefit in VSCC therapy.

Conclusions

In summary, although vulvar carcinoma does not present a diagnostic challenge, methods to predict the disease course in early-stage VSCC patients are missing. Considering the standard treatment of those patients with radical vulvectomy with bilateral inguofemoral lymphadenectomy which carries a significant burden of morbidity [62], there is an urgent need to better stratify early-stage patients. A tool optimizing surgical decisions, identifying patients who should not undergo lymphadenectomy and those who should receive additional treatment to the groin, would be highly beneficial.

Our study aimed to reveal novel mechanistic information on VSCC tumorigenesis and to identify potential biomarkers of disease progression through a proteomic approach. We identified disturbances in protein content both in cancer cells and their microenvironment within VSCC tumor. The biological insight from this discovery phase of the study points at inflammation as a key event during VSCC tumorigenesis. Indirect but numerous lines of evidence suggest that bacterial infections are the causative agent in vulvar cancer-related inflammation.

In early-stage VSCC, HMGA2 and PRTN3 should be considered as potential protein markers for the prediction of disease progression, as well as putative therapeutic targets. Furthermore, our results lay the foundation for the hypothesis that changes in the genital microbial flora may impair immune homeostasis during VSCC development and progression. Increased abundance of HMGA2 and PRTN3 in aggressive VSCC was found to be independent of the hrHPV status of the tumors. This further suggests that bacterial rather than viral infection status should be considered in the development of targeted

therapies in VSCC. Prevention and control of bacterial infections are anticipated to become a valuable addition to current cancer treatment modalities [63]. Finally, several lines of evidence for similarities in the biology of ANCA-associated vasculitis provides grounds for the assumption that VSCC patients might benefit from treatments currently applied in AVV and other neutrophilic human diseases such as COPD.

Materials And Methods

Patients

Clinical material was obtained from patients undergoing surgery for VSCC, from patients with premalignant vulvar lesions (HSIL and dVIN) (tissue and blood samples) and from healthy volunteers (blood) at the Maria Skłodowska-Curie National Research Institute of Oncology in Warsaw and at the Holycross Cancer Center in Kielce, Poland, between January 2002 and February 2019. Patients with microscopically confirmed VSCC at early (85 FIGO stage I, 7 FIGO stage II) and advanced stages (51 FIGO stage III, 2 FIGO stage IV) as well as patients with local recurrence (n = 17) were enrolled. Additionally, 14 patients with vaginal wall prolapse who underwent routine plastic surgery of the reproductive organ at the Holy Family Hospital in Warsaw, Poland, between 2014 and 2015 were assigned to the control group.

Samples

The numbers of samples, according to the stages of the study, are provided in (Fig. S4). At the discovery phase, the samples were included retrospectively. Primary VSCC tumors samples were divided into two separate categories depending of the subsequent disease course. The first comprised tumors samples of patients that were found disease-free during the follow-up period (“d-fVSCC”) and the second – tumors samples obtained from patients who progressed (“progVSCC”). The progression was defined as local recurrence, regional lymph node recurrence or cancer-related death. In addition, tumor samples obtained during vulvectomies of patients with primary VSCC enrolled prospectively (prosVSCC) and of patients with local VSCC recurrence (recVSCC) were included at the successive stages of the study. Finally, plasma samples were used for targeted protein quantitation in the blood of VIN and VSCC patients. Normal vulvar tissue samples and sera of healthy female volunteers served as the control samples (C) for the relevant stages of the study. There was only a partial overlap in the samples in the consecutive the stages of the study due to limited numbers of specimens available for analyses.

The hrHPV status of tissue samples was determined, as described previously [64], using the AmpliSens® HPV HCR-genotype-titre-FRT kit (InterLabService Ltd.), which detects 14 high risk HPV (hrHPV) genotypes, namely: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68.

Detailed descriptions of methodology can be found in Supplementary Materials.

Abbreviations

AAV

ANCA-associated vasculitis; adjp:adjusted p-value; AJCC:American Joint Committee on Cancer; ANCA:anti-neutrophil cytoplasmic antibody; AUC:area under receiver operating characteristics (ROC) curve; COPD:chronic obstructive pulmonary disease; CTSG:cathepsin G; DEP:differentially expressed protein; dVIN:differentiated-type vulvar intraepithelial neoplasia; ELANE:neutrophil elastase; FC:fold change; FIGO:International Federation of Gynecology and Obstetrics; GPA:granulomatosis with polyangiitis and vasculitis; H&E:hematoxylin and eosin staining; HMGA2:High Mobility Group AT-Hook 2; hrHPV:high-risk human papilloma virus; HSIL:high grade squamous intraepithelial lesion; IHC:immunohistochemistry; IPA:Ingenuity Pathway Analysis; iTRAQ:isobaric tags for relative and absolute quantitation; KEGG:Kyoto Encyclopedia of Genes and Genomes; MDS:myelodysplastic syndrome; MPO:myeloperoxidase; MS:mass spectrometry; NET:neutrophil extracellular trap; NLR:neutrophil-to-lymphocyte ratio; NSP:neutrophil serine protease; PFS:progression-free survival; PLR:platelet-to-lymphocyte ratio; PR-3:neutrophil serine proteinase 3; PRM:parallel reaction monitoring; PRTN3:proteinase 3; VIN:vulvar intraepithelial lesions (both dVIN and HSIL); VSCC:squamous cell vulvar carcinoma (progVSCC: VSCC tumors from patients who progressed, d-fVSCC:VSCC tumors from disease-free patients during follow-up; recVSCC:local VSCC recurrence).

Declarations

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Authors' contributions

MK conceived the study and sought funding. Proteomic experiments were designed and performed by AF and DD. NR performed verification experiments (immunohistochemical and ELISA analyses). EB-Z performed histological evaluation of the samples. AK, AW and KZ collected clinical specimen and interpreted the clinical results. SZ performed the microorganism detection and the data analysis. MK, AK and KG supervised data acquisition and performed data analyses. MK and DD wrote the paper with input from all authors, who approved the final version of the manuscript.

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Availability of data and materials

All data generated during this study are included either in article or in the additional files. Global proteomic data will be uploaded to the PRIDE database upon article acceptance.

Ethics approval and consent to participate

The study received ethical institutional board approvals of the Maria Sklodowska-Curie Institute - Oncology Center (No. 44/2002, 16/2015), Holycross Cancer Center (No. 15/2014) and Holy Family Hospital (2014-11-26).

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Top ten Canonical pathways enriched in progVSCC compared to d-fVSCC tumors, identified by core analysis in Ingenuity Pathway Analysis (IPA). (A) Top ten Canonical pathways enriched by IPA. The vertical orange line represents the threshold p-value (0.05) for pathway's enrichment. The horizontal axis is the $-\log(p \text{ value})$. Pathways predicted to be activated are in orange (based on the Z-scores, the activation scores determined by IPA), and grey bars relate to pathways with no activity pattern available.

(B) Overlapping canonical pathways that were significantly enriched in the dataset. Nodes correspond to canonical pathways whereas links correspond to the molecules shared between two pathways (no line means no shared molecules between two pathways). The color intensity of nodes corresponds to the $-\log p$ -value (a more intense color indicates a larger $-\log p$ -value).

Figure 2

The percentage of HMGA2-positive neoplastic cells. Examples of (A) immunohistochemical HMGA2 staining performed on tissue sections of HSIL, (B) progVSCC and (C) recurrent VSCC tumor samples. Upper panel - hematoxylin and eosin stained (H&E) specimen; middle panel – HMGA2 immunostained sections. Images were taken at 40x magnification. Scale bar, 100 μ m. (D) Comparison of the IHC results for VIN (HSIL and dVIN; n=50) and VSCC (n=39). (E) The same comparison when these two sample sets were subdivided into (HSIL; n=44 and dVIN; n=6); d-fVSCC (n=19), progVSCC (n=13) and recVSCC (n=7) samples. f, Survival curves (time to progression) according to the relative numbers of HMGA2-positive cancer cells in primary VSCC tumors (n=31). Abbreviations: PFS – progression-free survival; high and low – high and low proportion of HMGA2-positive cancer cells (≥ 30 and ≤ 10 cells, respectively).

Figure 3

The results of PRTN3 immunostaining. Examples of immunohistochemical PRTN3 staining performed on tissue sections of (A) HSIL and (B, C, D) VSCC tumors. High numbers of macrophages can be seen in b and c specimens, whereas the d sample contains PRTN3-positive cancer cells. Images were taken at 20x (A-C) and 40x magnification (D). The percent of PRTN3-positive neoplastic cells (E), macrophages (F) and lymphocytes (G) in VIN (HSIL; n=40 and dVIN; n=5), d-fVSCC (n=34), progVSCC (n=31) and recurrent

VSCC (n=3) samples. Survival curves (time to progression) according to the proportion of PRTN3-positive (H) cancer cells, (I) macrophages and (J) lymphocytes in primary VSCC tumors (n=64). Abbreviations: PFS – progression-free survival; high and low – high and low proportions of PRTN3-positive cells.

Figure 4

(A) PRTN3 and (B) ANCA blood levels. The results for the sera of 37 VIN patients (HSIL, n=27; dVIN, n=7), patients with primary (n=64) and recurrent VSCC (n=10) were compared to the control sera obtained from healthy women (C, n=44). Survival curves (time to progression) according to the serum levels of (C) PRTN3 and (D) ANCA in patients with primary VSCC (n=64). Abbreviations: PFS - progression-free survival; high - high protein level, i.e. above the cut-off value; low - protein level, i.e. below the cut-off value (i.e. the median of protein concentration).

Figure 5

Distribution of bacterial species detected in VSCC samples. Each column corresponds to the individual tumor case (d-fVSCC, n=16; progVSCC, n=16 and prosVSCC, n=3). Each row corresponds to an individual microorganism with its presence or absence depicted in green or grey, respectively. The results of genotyping of 13 vaginal microorganisms is related to hrHPV status of a given tumor with the virus presence or absence depicted in blue or grey, respectively. The values on the y-axis describe the number (0-5) of detected microorganisms per sample, whereas the values on x-axis describe the number (0-20) and a percentage of the samples with the presence of a given microorganism.

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