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Somatic mutation profiling of vulvar cancer: Exploring therapeutic targets

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HIGHLIGHTS

- Vulvar tumors, divided into high risk (hr) HPV(+) and hrHPV(−) groups, were screened for mutations in 50 genes by NGS.
- Mutations were detected at comparable frequencies in the two tumor groups.
- Mechanisms of two routes of VSCC pathogenesis may be similar despite being initiated from different premalignant lesions.
- Most of the identified mutations may activate the PI3K/AKT/mTOR pathway.
- The confirmed contribution of mTOR protein to vulvar cancer progression points towards putative therapeutic targets.

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ABSTRACT

Background. Vulvar squamous cell carcinoma (VSCC) constitutes over 90% of vulvar cancer. Its pathogenesis can follow two different pathways; high risk human papillomavirus (hrHPV)-dependent and HPV-independent. Due to the rarity of VSCC, molecular mechanisms underlying VSCC development remain largely unknown. The study aimed to identify pathogenic mutations implicated in the two pathways of VSCC development.

Methods. Using next generation sequencing, 81 VSCC tumors, 52 hrHPV(+) and 29 hrHPV(−), were screened for hotspot mutations in 50 genes covered by the Ion AmpliSeq Cancer Hotspot Panel v2 Kit (Thermo Fisher Scientific).

Results. Mutations of *TP53* (46% and 41%, of hrHPV(+) and hrHPV(−) cases respectively) and *CDKN2A* (p16) (25% and 21%, of hrHPV(+) and hrHPV(−) cases respectively) were the most common genetic alterations identified in VSCC tumors. Further mutations were identified in *PIK3CA*, *FBXW7*, *HRAS*, *FGFR3*, *STK11*, *AKT1*, *SMAD4*, *FLT3*, *JAK3*, *GNAQ*, and *PTEN*, albeit at low frequencies. Some of the identified mutations may activate the PI3K/AKT/mTOR pathway. The activation of mTOR was confirmed in the vast majority of VSCC samples by immunohistochemical staining.

Conclusions. Detecting pathogenic mutations in 13/50 genes examined at comparable frequencies in hrHPV(+) and hrHPV(−) tumors suggest that genetic mechanisms of the two routes of VSCC pathogenesis may be similar, despite being initiated from different premalignant lesions. Importantly, our data provide a rationale for new anti-VSCC therapies targeting the PI3K/AKT/mTOR pathway.

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1. Introduction

Vulvar squamous cell carcinoma (VSCC) accounts for over 90% cases of vulvar cancer. It originates from the progression of high-grade squamous intraepithelial lesions (HSIL, former usual type neoplasia - uVIN) and differentiated-type vulvar intraepithelial neoplasia (dVIN). HSIL is almost exclusively associated with human papilloma virus (HPV) infection and HPV vaccination has been shown to prevent infection and thus also HSIL [1]; while in d-VIN HPV is detected only occasionally [2]. Despite histological differentiation, dVIN exhibits high oncogenic potential with increased progression risk compared to HSIL. HPV positivity accounts for approximately one third of vulvar carcinomas [3]. The mechanism leading to the progression of differentiated VIN or, as suggested by some authors, of lichen sclerosus (LS) to VSCC is not precisely known [4]. Two different types of VSCC are generally believed to arise from their own associated premalignant lesions following HPV-dependent and HPV-independent carcinogenesis pathways [5].

Rates of molecular changes (e.g. loss of heterozygosity or *TP53* mutations) are much higher in VSCC cases without oncogenic HPV infection than in those being HPV-positive [6, 7]. In contrast to the majority of HPV-positive tumors, a significant proportion (50–70%) of HPV-independent VSCC cases exhibit *TP53* mutations and TP53 protein overexpression [4, 8]. The presence of *TP53* mutations in dVIN and LS [8, 9], as well as the immunohistochemical detection of TP53 protein in 90% of dVIN, indicates that these mutations play an important role in the development of vulvar cancer [4, 10]. A recent literature review of the genetic and epigenetic changes in VSCC and its precursor lesions support the notion of two pathways for VSCC pathogenesis, in which HPV infection and *TP53* mutations play key and almost independent roles [8, 11].

The study aimed to identify pathogenic mutations implicated in the two pathways of VSCC development in high risk HPV-positive (hrHPV (+)) and high risk HPV-negative (hrHPV(−)) VSCC tumors using the next-generation sequencing method (NGS).

2. Materials and methods

2.1. Patients

Clinical material was obtained from 81 patients treated for primary (n = 78) and recurrent (n = 3) VSCC at the Maria Sklodowska-Curie Institute – Oncology Center in Warsaw, Poland and at the Holycross Cancer Center in Kielce, Poland between January 2003 and January 2018. Patients with microscopically confirmed VSCC at both early (49 FIGO stage I, 4 FIGO stage II) and advanced stages (27 FIGO stage III, 1 FIGO stage IV) were enrolled. The collected primary tumor samples were frozen in liquid nitrogen and stored at −70 °C until DNA isolation. The hematoxylin-eosin stained slides corresponding to the collected snap-frozen tissues were initially reviewed by an expert gynecological pathologist, to whom the subsequent molecular and immunohistochemical results were blinded. Paraffin-embedded tissue specimens obtained from 41 patients with VSCC (32 primary tumors and nine recurrent) and 49 patients with premalignant vulvar lesions (43 with HSIL and six with dVIN) were investigated by immunohistochemical analysis. Research ethics board approval was received for the study and institutional authorization of the Maria Sklodowska-Curie Institute – Oncology Center (No. 44/2002, 16/2015) and Holycross Cancer Center (No. 15/2014). All patients gave their informed consent and all methods were performed in accordance with the relevant guidelines and regulations.

2.2. Cell lines

The human vulvar squamous cell carcinoma (VSCC) cell line A431 (DSMZ, Germany) was cultured in DMEM (Dulbecco's modified Eagle's medium, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific), and 1% penicillin/streptomycin antibiotics (AB, Thermo Fisher Scientific). The CAL-39 VSCC cell line (DSMZ, Germany) was cultured in DMEM supplemented with 10% heat inactivated FBS (Thermo Fisher Scientific), 2 mM L-glutamine (Thermo Fisher Scientific), 0.5 nM hydroxycortisole (Sigma-Aldrich), 10 ng/ml EGF (Sigma-Aldrich) and 1% AB. Both cell lines were cultured in a monolayer, under humidified conditions at 37 °C in 5% CO_2. SW-954 VSCC cell line (ATCC, USA) was cultured in Leibovitz L-15 Medium (Thermo Fisher Scientific) supplemented with 10% FBS and 1% AB. The SW-954 cells were cultured in a monolayer under humidified conditions in 100% air.

2.3. DNA isolation

Genomic DNA was isolated from approximately 25 mg of each tissue sample pulverized with a Microdismembrator II (B Braun Biotech International) or from 10^7 cultured cells using the NucleoSpin Tissue kit (Macherey Nagel, Inc.), according to the manufacturer's protocol.

2.4. HPV genotyping

The eighteen HPV types were considered for this study as high-risk (hrHPV(+)), HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82, and probable high-risk types, 26, 53 and 66, based on the HPV classification proposed for cervical cancer by Muñoz et al. [12]. Twelve HPV low-risk types, i.e. HPV6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and CP6108 [12], as well as HPV55 of undetermined risk, were classified as being hrHPV(−). The HPV status was determined as previously [13], using the Linear Array HPV Genotyping Test and Linear Array HPV Detection Kit (Roche Molecular Systems, Inc.), detecting 37 HPV genotypes, namely: HPV6, 11, **16**, **18**, 26, **31**, **33**, **35**, **39**, 40, 42, **45**, **51**, **52**, 53, 54, 55, **56**, **58**, **59**, 61, 62, 64, **66**, 67, **68**, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39, and CP6108 (high-risk in bold). Testing for the presence of hrHPV was also performed by AmpliSens HPV HCR-genotype-titre-FRT test (InterLabService Ltd.), which detects 14 hrHPV genotypes, namely HPV**16**, **18**, **31**, **33**, **35**, **39**, **45**, **51**, **52**, **56**, **58**, **59**, **66**, and **68**, following the manufacturer's instructions. The concordance index of the hrHPV genotyping results was assessed by Cohen's kappa coefficient using GraphPad Prism (La Jolla, CA, USA).

2.5. Next generation sequencing (NGS)

2.5.1. Library preparation

10 ng of DNA from each sample was added to the multiplex PCR reaction for library preparation using the Ion AmpliSeq Library Kit 2.0, Ion AmpliSeq Cancer Hotspot Panel v2 Kit (CHPv2), according to the manufacturer's instructions (Thermo Fisher Scientific). CHPv2 contains 207 pairs of primers, covering hotspots in the following 50 genes: *ABL1*, *EZH2*, *JAK3*, *PTEN*, *ACT1*, *FBXW7*, *IDH2*, *PTPN11*, *ALK*, *FGFR1*, *KDR*, *RB1*, *APC*, *FGFR2*, *KIT*, *RET*, *ATM*, *FGFR3*, *KRAS*, *SMAD4*, *BRAF*, *FLT3*, *MET*, *SMARCB1*, *CDH1*, *GNA11*, *MLH1*, *SMO*, *CDKN2A*, *GNAS*, *MPL*, *SRC*, *CSF1R*, *GNAQ*, *NOTCH1*, *STK11*, *CTNNB1*, *HNF1A*, *NPM1*, *TP53*, *EGFR*, *HRAS*, *NRAS*, *VHL*, *ERBB2*, *IDH1*, *PDGFR*, *ERBB4*, *JAK2*, *PIK3CA*. The products with a mean length of 156 bp (range 111–187 bp) obtained by the multiplex PCR were subjected to partial enzymatic digestion with FuPa reagent (Ion AmpliSeq Library Kit 2.0, Thermo Fisher Scientific) to remove the primers. The adapters were then enzymatically attached to the multiplex PCR products using the Ion Xpress Barcode Adapters 1–32 Kit (Thermo Fisher Scientific). One of the adapters contained a unique barcode sequence, which allowed identification of sequences derived from any given patient within a mixture of libraries. The prepared libraries were purified by two rounds of Agencourt AMPure XP (Beckman Coulter Genomics), according to the instructions of the manufacturer of the Ion AmpliSeq Library Kit (Thermo Fisher Scientific).

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