LRIG1-2 and LMO7 immunoreactivity in vulvar squamous cell carcinoma: Association with prognosis in relation to HPV-DNA and p16^{INK4a} status

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Abstract. The present study was conducted to investigate the possible prognostic value of molecular markers LRIG1-2 and LIM domain 7 protein (LMO7) in vulvar squamous cell carcinoma (VSCC) and their possible correlation to human papilloma virus (HPV)- and p16^{INK4a}-status of the tumors. Patients diagnosed with VSCC at the University Hospital of Umeå, Sweden, during the years 1990-2013 were selected. Tumor blocks were retrieved from tissue archives and clinical data were collected from the records of patients. HPV-PCR analysis, HPV genotyping and immunohistochemistry were performed. In total, 112 patients were included. Forty percent of the tumors were HPV-positive, 27% were p16^{INK4a}-positive and 23% were positive for both HPV and p16^{INK4a} (considered HPV-driven). HPV-positivity and p16^{INK4a}-positivity were associated with prolonged disease-free survival (DFS) in Kaplan-Meier survival analysis. Leucine-rich repeats and immunoglobulin-like domains 1 (LRIG1) immunoreactivity was not significantly associated with survival. High leucine-rich repeats and immunoglobulin-like domains 2 (LRIG2) immunoreactivity was associated with a prolonged overall survival (OS) (P=0.001). By analyzing HPV-negative cases only, it was determined that high LRIG2 immunoreactivity was associated with both favorable OS (P=0.008) and DFS (P=0.031). LRIG2 immunoreactivity was also an independent prognostic factor in multivariate analysis of OS (P=0.002, HR=0.41; 95% CI, 0.24-0.71). High immunoreactivity with LMO7-1250 antibody was associated with survival benefits in the whole cohort (OS; P=0.011) although DFS was only prolonged in HPV-negative and not HPV-driven tumors (P=0.038 and 0.042, respectively). The present study indicated that LRIG2 and LMO7 may be useful prognostic markers in VSCC, particularly for patients without HPV-driven tumors or with advanced tumors at diagnosis. In contrast to earlier observations regarding other types of squamous cell carcinoma, LRIG1 was not a significant prognostic factor in VSCC.

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Introduction

Vulvar cancer is a relatively rare disease, accounting for 4% of gynecological malignancies globally. The majority of vulvar cancer cases are of squamous cell carcinoma (SCC) histology. The incidence of vulvar cancer has increased in several countries during the last decades, particularly among younger women (1). In Sweden, however, the incidence remains at ~3/100,000 women since the 1990s (2). Vulvar SCC (VSCC) can be classified as human papilloma virus (HPV)-independent and HPV-dependent. HPV-independent tumors usually arise later in life and are often preceded by long-standing vulvar dermatosis (3). The frequency of high-risk HPV-positive VSCC is reported to be between 28.6 and 44.7%. The most common HPV-subtype in VSCC is HPV16 (4,5). The proposed pathologic mechanism in HPV-dependent tumors is inactivation of tumor suppressor proteins p53 and Rb by the HPV-derived oncogenic proteins E6 and E7, causing alteration of signal transduction pathways to promote transformation (6). In HPV-transformed cells, the p16^{INK4a}/CDK4/pRB pathway is blocked resulting in accumulation of the cyclin-dependent kinase-4 inhibitor p16^{INK4a} (3). Overexpression of p16^{INK4a} has been viewed as a pseudo-marker for high-risk HPV-infection (7). However, according to the study by de Sanjosé *et al* only 87.9% of HPV-positive invasive VSCC are positive for p16^{INK4a} (4). This study as well as another (5), suggest that only tumors with the combined presence of HPV-DNA and p16^{INK4a} overexpression should be viewed as truly HPV-driven. HPV- and/or p16^{INK4a}-positive tumors of female genitalia, as well as head and neck tumors, have been associated with significant survival benefits compared to HPV- and p16-negative tumors (5,8,9).

This study focused on the expression of the leucine-rich repeats and immunoglobulin-like domains (LRIG) family of transmembrane proteins and the LIM domain 7 protein (LMO7) in VSCC. The LRIG protein family includes three members in humans, LRIG1, LRIG2 and LRIG3 (10-12). LRIG1 is the most studied family member and has been revealed to negatively regulate several oncogenic receptor tyrosine kinases including EGFR, ERBBs 2-4, MET, RET and PDGFR-A (13). Substantial evidence suggests that LRIG1 functions as a tumor suppressor in various contexts (13,14). LRIG1 was revealed to be a positive prognostic factor in cervical SCC and cervical adenocarcinoma (13). Less is known about the functions and prognostic values of LRIG2 and LRIG3. LMO7 has been revealed to interact with LRIG1 and LRIG3 (15). LMO7 is a proposed stabilizer of adherence junctions and transcription factor for muscle related genes. It has also been associated with different human cancers (16-18). Loss of LMO7 in a mouse model led to spontaneous lung adenocarcinomas (19) and low expression of LMO7 in human lung adenocarcinomas has been reported to be associated with poor prognosis (16). Conversely, in another study, high expression of LMO7 was a negative prognostic factor in LRIG1 expressing non-small cell lung cancers (NSCLC) (20).

There are a few prognostic factors in VSCC, of which FIGO-stage and lymph node status are the most important (21). In a recent systematic review investigating known prognostic factors in VSCC, results were contradictory. Hence, there is a need for additional prognostic factors for clinical decision-making in VSCC (22). The aim of the present study was to investigate possible prognostic values of LRIG1-2 and LMO7 in VSCC and their possible association to HPV- and $p16^{INK4a}$-status in tumors of patients from northern Sweden.

Materials and methods

Patients and specimens. Patients diagnosed with VSCC in the northern region of Sweden between 1990 and 2013 were identified through the Swedish Cancer Registry. Out of 258 correctly classified patients treated at the University Hospital in Umeå, 34 declined to participate, 81 were excluded due to missing clinical data and in 31 cases material could not be obtained. Finally, this study was based on 112 patients with an age range of 37-94 years. Patients' records were retrieved from the Department of Oncology at the University Hospital in Umeå and clinical data were collected. Formalin-fixed, paraffin-embedded (FFPE) specimens from diagnostic biopsies or resection material from the time of diagnosis were retrieved from the biobank at Västerbotten County Council (Umeå, Sweden). FFPE-material was handled and stored in room temperature. All patients alive at the start of the study signed informed consent for the use of their tissues. This research was approved by the Regional Ethical Review Board at Umeå University.

HPV DNA analysis. DNA was extracted from 25-30 μm FFPE sections using the QIAamp DNA Tissue FFPE Tissue kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. To check for possible contamination, a negative control consisting of an empty test tube was included between every fifth sample and treated likewise. DNA-samples were stored at 20˚C.

HPV-PCR analysis was carried out using 125 ng of extracted DNA with the general primer pair GP5+/6+, amplifying a fragment of the conserved HPV L1 gene (23). Primer sequences were as follows: Forward, 5'-TTTGTTACTGTGGTAGAT ACTAC-3' and reverse, 5'-GAAAAATAAACTGTAAATCAT ATTC-3'. The 50 μl PCR mixture consisted of 5 μl GeneAmp 10X PCR Gold buffer, 3.5 mM $MgCl_2$ (both from Applied Biosystems Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 200 μM of each dNTP (GeneAmp dNTP mix) (Thermo Fisher Scientific, Inc.), 25 pmol of each primer and 1 unit of AmpliTaq Gold DNA Polymerase (Applied Biosystems Life Technologies; Thermo Fisher Scientific, Inc.). Amplification was performed in a Biometra professional thermocycler (Thermo Fisher Scientific, Inc.) or a T100 thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The reaction was initiated with denaturation for 4 min at 94˚C, followed by 40 amplification cycles of denaturation at 94˚C for 1 min, annealing at 44˚C for 1 min and elongation at 72˚C for 2 min. The final cycle ended with a prolonged elongation step at 72˚C for 10 min. PCR products were run on a 2% agarose gel in 50 mM Tris/37 mM Borate/1.3 mM EDTA and stained with 0.5X GelRed (Biotium, Inc., Hayward, CA, USA). Gels were visualized under UV-light. Fragments of 130-150 bp were considered HPV-positive.

To identify samples that were incorrectly classified as HPV-negative when using the GP5+/6+ primer pair due to disruption of the L1 gene, GP5+/6+ -negative samples were re-analyzed using the general primer pair CpI/IIG (24). Primer sequences were as follows: Forward, 5'-TTATCWTAT GCCCAYTGTACCAT-3' and reverse, 5'-ATGTTAATWSAG CCWCCAAAATT-3'. The 50-μl PCR mixture consisted of 5 μl GeneAmp 10X PCR Gold buffer, 200 μM of each dNTP (GeneAmp dNTP mix), 3 mM $MgCl_2$, 17 pmol CpI, 26 pmol CpIIG, and 1 unit of AmpliTaq Gold DNA Polymerase. The amplification consisted of denaturation for 5 min at 94˚C, followed by 40 amplification cycles of denaturation at 95˚C for 1 min, annealing at 55˚C for 1 min and elongation at 72˚C for 2 min. The final cycle ended with a prolonged elongation step at 72˚C for 4 min. Samples with products of ~188 bp were considered positive.

To assess the quality of the DNA-preparations, samples negative for both primer pairs were run on PCR using the s14 sense/antisense primers (25). Primer sequences were as follows: Forward, 5'-TCGAAAGGGGAAGGAAAAGA-3' and reverse, 5'-CAGTGACATGGACAAAAGTG-3'. The 50-μl PCR mixture consisted of 5 μl GeneAmp 10X PCR Gold buffer, 200 μM of each dNTP (GeneAmp dNTP mix), 1.5 mM $MgCl_2$, 15 pmol of each primer and 1 unit of AmpliTaq Gold DNA Polymerase. The amplification consisted of denaturation for 1 min at 94˚C, followed by 40 amplification cycles of denaturation at 94˚C for 30 sec, annealing at 50˚C for 30 sec and elongation at 72˚C for 45 sec. The final cycle ended with a prolonged elongation step at 72˚C for 5 min. Samples with products of ~127 bp were considered to contain amplifiable DNA.

Genotyping was performed using the PapilloCheck® HPV screening-kit and the CheckScanner™ laser scanner (Serial no. 700x0177) (Greiner Bio-One North America Inc., Monroe, NC, USA). In brief, this method detects 24 different HPV types through PCR amplification of a 350-bp fragment of the E1 gene and hybridization to specific DNA probes on a DNA

chip. The HPV-types detected by the assay included eighteen high-risk types (16, 18, 45, 31, 33, 52, 58, 35, 59, 56, 51, 39, 68, 73, 82, 53, 66 and 70) and six low-risk types (6, 11, 40, 42, 43 and 44/55).

Immunohistochemistry. FFPE sections (4 μm) were deparaffinized, rehydrated, and rinsed in water. Immunohistochemistry was performed using Ventana standard procedure on a Ventana BenchMark ULTRA instrument (Ventana Medical Systems, Inc., Tucson, AZ, USA). Antigen retrieval was performed with a CC1 buffer (Ventana Medical Systems, Inc.). Antibodies used were as follows: Rabbit anti-LRIG1 (product. no. AS184165; AgriSera AB, Vännäs, Sweden), 22 μg/ml; rabbit anti-LRIG2-151 (12), 3 μg/ml; rabbit anti-LMO7-1250 (15), 24 μg/ml; rabbit anti-LMO7 (cat. no. HPA020923; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 2 μg/ml; mouse monoclonal anti-p16^{INK4a} (E6H4) (Ventana Medical Systems, Inc.), 1 μg/ml. For validation of anti-LRIG1 and anti-LMO7 antibodies, please refer to Figs. S1 and S2, respectively.

Evaluation and classification of immunostaining. Evaluation of immunostainings was performed by a senior pathologist without knowledge of the disease outcome. Immunoreactivity was scored based on both staining intensity and percentage of immunoreactive epithelial cells within the tumor. Intensity was evaluated on a four-grade semi-quantitative scale; no staining, weak intensity, moderate intensity or strong intensity. For statistical purposes, intensity scores were grouped into no/weak and moderate/strong. The percentage of positive cells was grouped into 'at or above median' or 'below median' to obtain relatively even groups. For simplicity, at or above median is henceforth referred to as 'high' and below median as 'low' staining percentage. For p16^{INK4a} immunostaining, only cases with a strong nuclear and cytoplasmic expression in a continuous segment of at least 10-20 cells were considered positive.

Statistical analyses. Patient characteristics were analyzed with the independent sample t-test for comparison of means or the Pearson Chi-square test for ordinal variables. Two-sided P-values were reported. Disease-free survival (DFS) was defined from the date of diagnosis to the date of accession to the patients' records or the date of recurrence [according to pathological anatomical diagnosis (PAD) or the date of first recorded clinical progression]. Death without documented recurrence was censored at the date of death. Overall survival (OS) was defined as the time from the date of diagnosis to the date of death irrespective of cause. If a patient was still alive at the time of accessing the patients' records, the case was censored at that date. DFS and OS were illustrated in Kaplan-Meier graphs and a log-rank test was used for comparison of variables. In the figures presented, the x-axis was truncated at 120 months of follow-up. In the multivariate analysis, tumors were grouped into low and high percentage of positively-stained cells with a cut-off value at median. All parameters revealing a significant difference when comparing survival were included. The median age in each group was compared with the Mann-Whitney test. P-values <0.05 were considered to indicate a statistically significant difference. All statistical analyses were performed using the SPSS software (IBM SPSS Statistics for Windows, version 23; IBM Corp., Armonk, NY, USA). Due to failure of DNA preparation or immunohistochemical staining, occasional data were missing in various statistical analyses.

Results

Study population. The 112 patients diagnosed with VSCC during the period of 1990 to 2013 from whom clinical data and representative material could be obtained were included in the study. The cases that were excluded due to lack of material exhibited no significant difference compared to those that were included regarding age, FIGO-stage or histopathological grade.

Clinical characteristics. Mean age at diagnosis was 70 years. The mean age at diagnosis of patients with HPV-negative and HPV-positive tumors was 72 and 66 years, respectively. This difference was significant (P=0.008). HPV-negative and HPV-positive tumors differed significantly also regarding lichen sclerosus et atrophicus (LSA) in disease history (P=0.001) and rate of recurrence or persistence of disease (P=0.027). Within the HPV-negative group, LSA was more common, and recurrence was more likely to occur. Cases negative vs. positive for p16^{INK4a} also differed significantly, and in the same manner, regarding record of LSA in disease history (P<0.001) and rate of recurrence (P=0.027). Clinical characteristics in relation to HPV-status are summarized in Table I.

HPV and p16^{INK4a} analyses. Forty percent (44/109) of tumors were HPV-positive, 26% (26/99) were p16^{INK4a}-positive and 23% (22/97) were positive for both HPV and p16^{INK4a}. The latter category was referred to as HPV-driven in accordance with a recent hypothesis (4,5). Four tumors were HPV-negative and p16^{INK4a}-positive whereas 14 tumors were HPV-positive and p16^{INK4a}-negative. Fifty-seven cases were negative for both HPV and p16^{INK4a}. In three cases the DNA preparation was of insufficient quality and in 13 cases p16^{INK4a} data could not be retrieved. From a total of 44 HPV-positive samples, 41 were subjected to genotyping. Of these, 20 samples were successfully genotyped. Ten samples failed the internal control, probably due to insufficiency of material or quality of the sample. In eleven cases, internal control was satisfactory, however, the genotyping rendered a negative result. This may be explained by bad quality of the preparation or, alternatively, disruption of the E1 gene in the specific tumors. Among successfully typed tumors, HPV16 was the most prevalent type (13/20) and HPV33 the second most prevalent type (6/20). HPV42 was found in one of the samples positive for HPV33 and one sample contained HPV58 only (data not shown).

Associations between HPV- and p16^{INK4a}-statuses and rates of recurrence and DFS. Both HPV- and p16^{INK4a}-positivity were associated with a lower rate of recurrence: 34.1% (15/44) of HPV-positive cases and 60% (39/65) of HPV-negative cases had a recurrence or persistent disease, 26.9% of p16^{INK4a}-positive cases and 57.5% of p16^{INK4a}-negative cases had a recurrence or persistent disease, and 22.7% of HPV-driven cases and 57.9% of not HPV-driven cases had a recurrence or persistent disease (data not shown). HPV-positive and p16^{INK4a}-positive

Table I. HPV status in relation to patient and tumor characteristics.

Variables	Category	HPV-DNA-, n (%)	HPV-DNA+, n (%)	Total, n	P-value
Mean age at diagnosis	Years	72 (range, 37-94)	66 (range, 44-94)	70 (mean)	0.008[a]
Previous gynecological malignancy	Yes	3 (4.7)	1 (2.3)	4	0.644
	No	61 (95.3)	43 (97.7)	104	
Hysterectomy before diagnosis	Yes	10 (15.4)	5 (11.4)	15	0.778
	No	55 (84.6)	39 (88.6)	94	
Record of LSA prior to malignancy	Yes	35 (53.8)	9 (20.5)	44	0.001[a]
	No	30 (46.2)	35 (79.5)	65	
Histopathological grade	Unknown	2 (3.1)	1 (2.4)	3	0.844
	Poor	13 (20.0)	12 (28.6)	25	
	Moderate	36 (55.4)	22 (52.4)	58	
	Well	14 (21.5)	7 (16.7)	21	
FIGO-stage	I	23 (36.5)	11 (25)	34	0.558
	II	20 (31.7)	16 (36.4)	36	
	III	16 (25.4)	15 (34.1)	31	
	IV	4 (6.3)	2 (4.5)	6	
Local metastasis at diagnosis	Yes	16 (25)	16 (36.4)	32	0.284
	No	48 (75)	28 (63.6)	76	
Distant metastasis at diagnosis	Yes	1 (1.6)	0 (0)	1	0.405
	No	63 (98.4)	44 (100)	107	
Recurrence	Yes	29 (44.6)	12 (27.3)	41	0.027[a]
	No	26 (40.0)	29 (65.9)	55	
	Persistent disease	10 (15.4)	3 (6.8)	13	
Mean time to recurrence	Months	48 (range, 0-290)	67 (range, 0-269)	56	0.124
Vital status	Alive	23 (35.4)	22 (50)	45	0.130
Cause of death	Cancer	16 (24.6)	9 (20.5)	25	
	Other	14 (21.5)	11 (25)	25	
	Unknown	12 (18.5)	2 (4.5)	14	

[a]P<0.05. HPV, human papilloma virus; LSA, lichen sclerosus et atropicus.

patients also exhibited a longer DFS than HPV-negative and $p16^{INK4a}$-negative patients (P=0.006 and P=0.010, respectively; Fig. 1B and C). Similarly, HPV-driven cases revealed a longer DFS than not HPV-driven cases (P=0.005) (Fig. 1D). However, no significant association between HPV- (P=0.152; Fig. 1A) or $p16^{INK4a}$-status and OS was observed.

LRIG immunohistochemistry. LRIG1 immunoreactivity was observed primarily in cell nuclei whereas LRIG2 immunoreactivity was primarily cytoplasmic and membraneous (Fig. 2A and B). When considering the percentage of immunoreactive tumor cells, there was a better clinical outcome for patients with a high proportion of LRIG-positive cells (Fig. 3). For LRIG1, this association was, however, neither significant when analyzing OS (Fig. 3A-D) nor DFS (data not shown). LRIG2 was significantly associated with a favorable OS but not DFS in the whole cohort (Fig. 3E; P=0.001 and P=0.051, respectively). Among the HPV-negative and not HPV-driven strata, LRIG2 was significantly associated with a favorable prognosis both regarding OS (P=0.008 and 0.001, respectively; Fig. 3F and G) and DFS (P=0.031 and 0.009, respectively). LRIG2 immunoreactivity was also an independent prognostic marker for OS in multivariate analysis (Table II).

When comparing intensity of LRIG1 divided into no/weak vs. moderate/high, no significant differences were observed between groups considering OS or DFS (data not shown). However, when stratified according to HPV-status, no/weak LRIG1 staining was associated with a better OS in HPV-negative cases (P=0.044) (data not shown). Stratifications into $p16^{INK4a}$-status, HPV-/not HPV-driven tumors or FIGO-stage provided no further significant findings. Intensity of LRIG2 computed in the same manner showed no significant differences between groups.

LMO7 immunohistochemistry. LMO7 immunostaining was evaluated using two different antibodies, one developed by our laboratory (LMO7-1250) and one commercially available (LMO7; Sigma-Aldrich; Merck KGaA). The former exhibited staining primarily in the nuclei whereas the latter stained predominantly around cell membranes and cytoplasmatically (Fig. 1C-E). High staining percentage with the LMO7-1250 antibody was significantly associated with a favorable OS, but

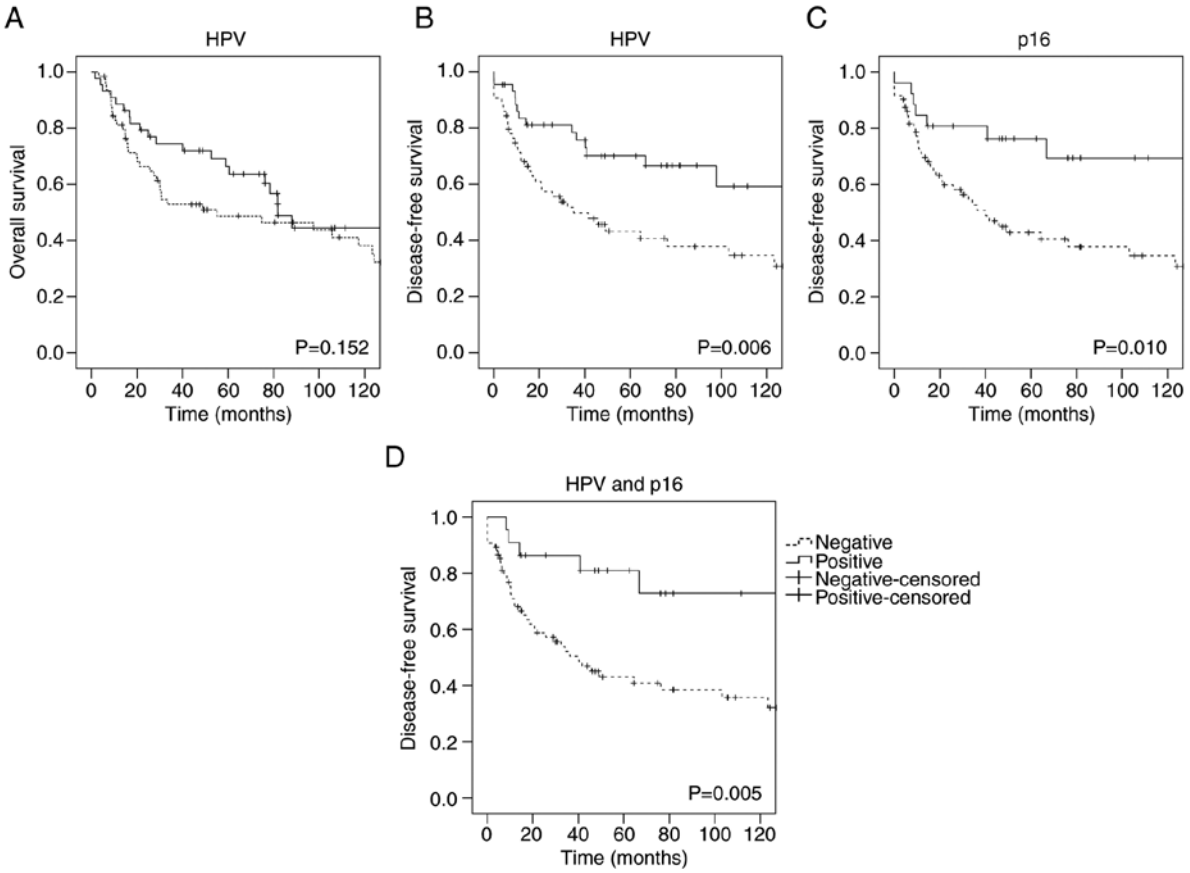

Figure 1. Kaplan-Meier survival curves revealing OS and DFS according to HPV- and p16^{INK4a}-status. (A) HPV-negative vs. HPV-positive tumors, OS, n=109. (B) HPV-negative vs. HPV-positive tumors, DFS, n=108. (C) p16^{INK4a}-negative vs. p16^{INK4a}-positive tumors, DFS, n=98. (D) Not HPV-driven vs. HPV-driven tumors, DFS, n=97. Of note, the not HPV-driven tumors were denoted negative and included all cases that were not concurrently HPV- and p16^{INK4a}-positive. OS, overall survival; DFS, disease-free survival; HPV, human papilloma virus.

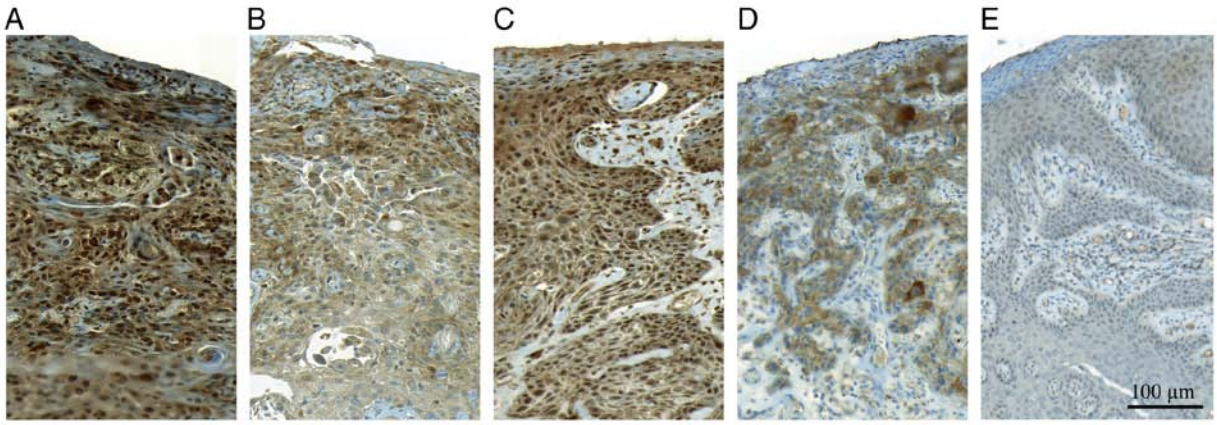

Figure 2. Microphotographs of immunohistochemical stainings. VSCC sections were labeled with hematoxylin nuclear counterstain (blue) and immunostained (brown) for (A) LRIG1, (B) LRIG2, (C) LMO7-1250, or (D) LMO7. (E) Negative control with antibody exchanged for rabbit IgG 100 μg/ml. Scale bar, 100 μm. VSCC, vulvar squamous cell carcinoma; LRIG1, leucine-rich repeats and immunoglobulin-like domains 1; LRIG2, leucine-rich repeats and immunoglobulin-like domains 2; LMO7, LIM domain 7 protein.

not DFS (data not shown), in the whole cohort (OS: P=0.011; DFS: P=0.139; Fig. 3I). In the HPV-negative and HPV-driven strata, the association was significant regarding both OS (P=0.021 and P=0.008, respectively; Fig. 3J and K) and DFS (P=0.038 and P=0.042, respectively). Additionally, among tumors with FIGO stage III-IV at diagnosis, high LMO7 staining percentage was associated with a favorable prognosis (P=0.011; Fig. 3L). The LMO7 antibody also revealed association with a favorable prognosis but only regarding OS and in the HPV-negative, p16^{INK4a}-negative and HPV-driven strata (data not shown). Similar to LRIG immunoreactivity results, evaluation of staining intensity produced inconclusive results.

Table II. Cox regression.

		Univariate analysis			Multivariate analysis		
	Variable	HR	95% CI	P-value	HR	95% CI	P-value
OS	Age (years)						
	<60	1	(ref)		1	(ref)	
	≥60	3.8	1.85-7.63	<0.001[a]	4.34	1.92-9.81	<0.001[a]
	FIGO						
	I + II	1	(ref)				
	III + IV	1.6	0.94-2.58	0.088			
	HPV-status						
	Negative	1	(ref)				
	Positive	0.7	0.40-1.12	0.125			
	p16						
	Negative	1	(ref)				
	Positive	0.7	0.37-1.25	0.212			
	LSA						
	No	1	(ref)				
	Yes	0.71	0.43-1.18	0.183			
	Local met						
	No	1	(ref)				
	Yes	1.46	0.87-2.46	0.155			
	LRIG1						
	Low	1	(ref)				
	High	0.71	0.43-1.20	0.202			
	LRIG2						
	Low	1	(ref)		1	(ref)	
	High	0.44	0.26-0.75	0.003[a]	0.41	0.24-0.71	0.002[a]
	LMO7-1250						
	Low	1	(ref)		1	(ref)	
	High	0.54	0.32-0.91	0.021[a]	0.63	0.36-1.10	0.104
	LMO7						
	Low	1	(ref)				
	High	0.68	0.41-1.15	0.149			
DFS	Age (years)						
	<60	1	(ref)				
	≥60	1.77	0.92-3.37	0.085			
	FIGO						
	I + II	1	(ref)		1	(ref)	
	III + IV	1.92	1.11-3.32	0.019[a]	2.00	1.12-3.60	0.020[a]
	HPV-status						
	Negative	1	(ref)		1	(ref)	
	Positive	0.43	0.24-0.78	0.006[a]	0.78	0.35-1.74	0.538
	p16						
	Negative	1	(ref)		1	(ref)	
	Positive	0.35	0.16-0.79	0.011[a]	0.29	0.12-0.68	0.005[a]
	LSA						
	No	1	(ref)				
	Yes	1.16	0.68-2.01	0.586			
	Local met						
	No	1	(ref)				
	Yes	1.65	0.95-2.87	0.076			
	LRIG1						
	Low	1	(ref)				

Table II. Continued.

		Univariate analysis			Multivariate analysis		
	Variable	HR	95% CI	P-value	HR	95% CI	P-value
DFS	High	0.49	0.46-1.45	0.490			
	LRIG2						
	Low	1	(ref)				
	High	0.059	0.33-1.02	0.059			
	LMO7-1250						
	Low	1	(ref)				
	High	0.67	0.38-1.19	0.169			
	LMO7						
	Low	1	(ref)				
	High	1.05	0.60-1.84	0.865			

[a]P<0.05. HR, hazard ratio; CI, confidence interval; HPV, human papilloma virus; LSA, lichen sclerosus et atropicus; LRIG1, leucine-rich repeats and immunoglobulin-like domain 1; LRIG2, leucine-rich repeats and immunoglobulin-like domain 1; LMO7, LIM domain 7 protein; OS, overall survival; DFS, disease-free survival.

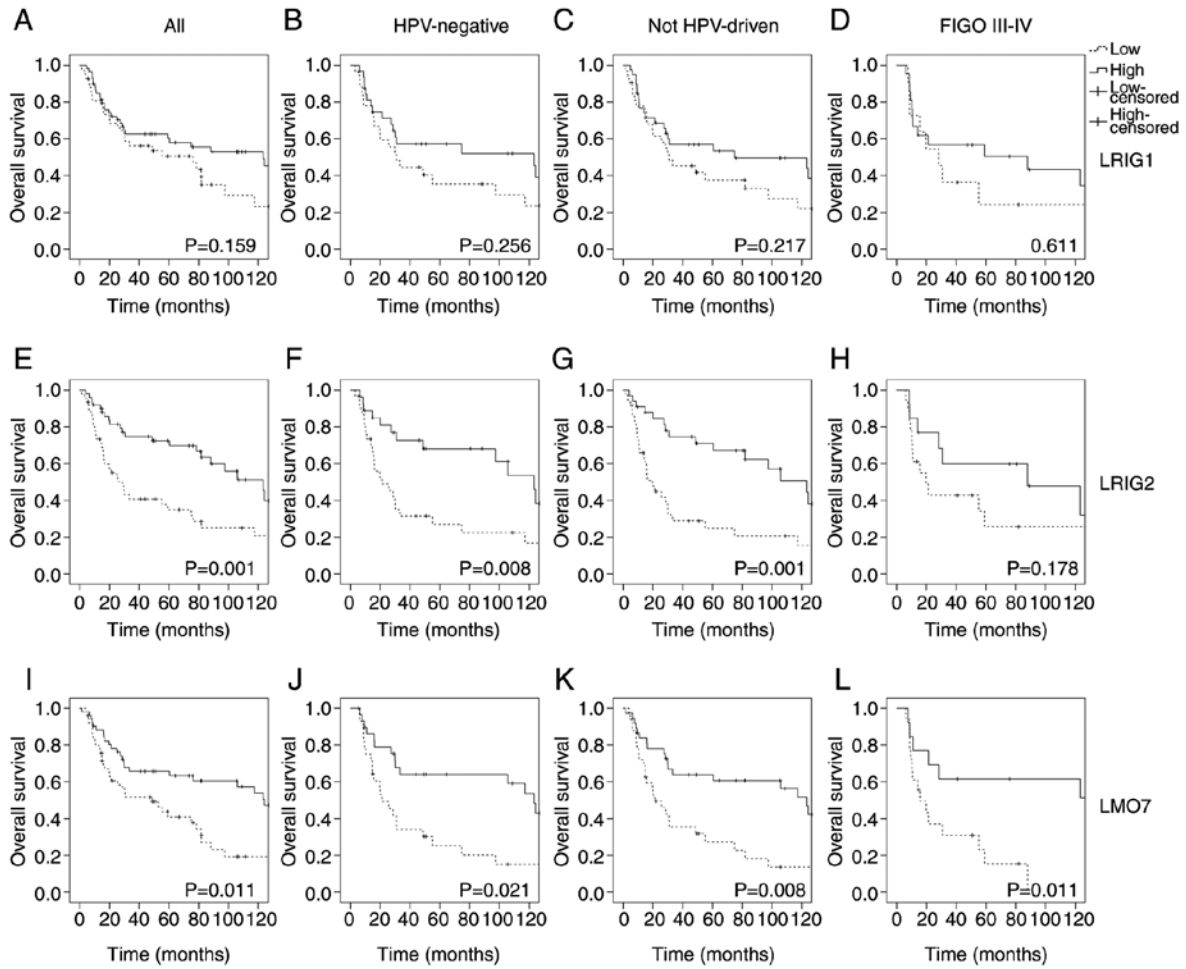

Figure 3. Kaplan-Meier survival curves revealing OS according to LRIG and LMO7-1250 immunoreactivities in the whole cohort and specified subgroups. Patients with tumors of high staining percentage were compared with patients with low staining percentage with a cut-off at median. (A) LRIG1, all tumors, n=101. (B) LRIG1, HPV-negative tumors, n=60. (C) LRIG1, not HPV-driven tumors, n=71. (D) LRIG1, FIGO stage III-IV, n=32. (E) LRIG2, all tumors, n=96. (F) LRIG2, HPV-negative tumors, n=58. (G) LRIG2, not HPV-driven tumors, n=69. (H) LRIG2, FIGO stage III-IV, n=31. (I) LMO7-1250, all tumors, n=101. (J) LMO7-1250, HPV-negative tumors, n=58. (K) LMO7-1250, not HPV-driven tumors, n=70. (L) LMO7-1250, FIGO stage III-IV, n=31. OS, overall survival; LRIG, leucine-rich repeats and immunoglobulin-like domain; LMO7, LIM domain 7 protein.

Discussion

In the present study, we evaluated the possible prognostic values of LRIG1-2 and LMO7 expression through immunohistochemical staining of FFPE material and collection of clinical data from 112 women diagnosed with VSCC in the northern region of Sweden. The results revealed that a high percentage of LRIG2-immunoreactive tumor cells was a significant and independent positive prognostic marker for OS. LRIG2 was also a significant positive prognostic factor when considering DFS in the HPV-negative tumors as well as in the not HPV-driven tumors, suggesting a disease-specific survival benefit of high LRIG2 expression in HPV-independent VSCC. This was in contrast to the results in invasive early stage cervical SCC, where high LRIG2 expression was correlated to poor survival (26) and in primary vaginal carcinoma where no significant correlation was revealed between LRIG2 and patient survival (27). Other studies have revealed LRIG2 to be a negative prognostic factor in oligodendroglioma (28) although a positive prognostic factor in glioblastoma/astrocytoma (29). LRIG1 was not a significant predictor of prognosis in VSCC in our study, although considerable evidence points to it being a positive prognostic marker in several other malignancies (13,20). Thus, results differ regarding the prognostic value of LRIG proteins in gynecological and other malignancies.

High LMO7 immunoreactivity was associated with better OS and DFS in our material. Similar to LRIG2, the association was stronger in HPV-independent tumors. Two antibodies reactive against LMO7 were used. Their different staining patterns may be explained by the different epitopes recognized by the antibodies and may reflect different splice variants of the protein or masking of epitopes due to post-translational modifications or protein binding. To the best of our knowledge, it is difficult to speculate upon how this would affect tumor progression although there may be a connection to LRIG function since LMO7 has been revealed to interact with both LRIG1 and LRIG3 in their endogenous form. LMO7 may also interact with LRIG2 although this has not been determined. Notably, their protein-protein interaction site is believed to be the LIM-domain in the C-terminal end of the LMO7-protein, which is the part recognized by the LMO7-1250 antibody (15). It is, however, still not possible to draw any clear conclusions about the possible mechanistic connections that may exist between LRIG proteins, LMO7 and HPV- and p16 status.

Our results revealed that both HPV- and $p16^{INK4a}$-positivity conferred a more favorable prognosis in VSCC. This has been previously revealed (5,30) and was consistent with findings in other HPV-related cancers (9,31-33). Tumors with combined HPV- and $p16^{INK4a}$-positivity exhibited an even stronger association with a favorable prognosis. This was consistent with the hypothesis proposed by de Sanjosé *et al* according to which the presence of HPV in the absence of $p16^{INK4a}$ overexpression may be due to a transient HPV infection, not contributing to carcinogenesis (4,34).

In conclusion, patients with HPV-independent VSCC had a survival deficit compared to HPV-dependent disease, and our data suggested a role for LRIG2 and LMO7 as positive prognostic factors among the HPV-independent cases, and LMO7 among the most advanced tumors. Thus, these markers could possibly provide means to facilitate selection of individual treatment strategies among VSCC patients. However, more research is warranted to further elucidate the functions and prognostic values of the studied molecular markers in VSCC.

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Availability of data and material

The datasets analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

KS carried out the DNA-preparations and PCR-analyses, gathered the approvals and clinical data of patients, assembled and analyzed the data statistically, wrote the manuscript and designed the figures. HO classified the tumors according to histology and differentiation grade, and evaluated the immunohistochemical stainings. CÖ assisted in statistical analysis and interpretation of the results. EL and HH provided support and the materials, helped supervise the study, critically read the manuscript and suggested changes. DL conceived and designed the study with the substantial contribution of EL. All authors critically read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The study was approved by the Regional Ethical Review Board at Umeå University, Umeå, Sweden (Dnr 2013-416-31M). Written informed consent was obtained from all participants alive at the start of the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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