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miRNA-expression in tonsillar squamous cell carcinomas in relation to HPV infection and expression of the antileukoproteinase SLPI

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ABSTRACT

The aim of this study was to determine if micro-(mi-)RNAs are involved in the previously reported inverse correlation between the antileukoproteinase SLPI, HPV, and smoking habit of head and neck squamous cells carcinoma (HNSCC) patients.

HPV-status and SLPI-protein expression were determined in tonsillar SCC (TSCC; n=126). Differentially expressed miRNAs dependent on HPV-status and SLPI-expression were detected by microarray; possible binding-sites in SLPI- and HPVE6-mRNAs were determined *in silico*. Survival rates were estimated testing prognostic values of HPV-status, SLPI- and miRNA-expression.

miRNA-array identified 24 up-regulated and 10 down-regulated miRNAs in HPV-positive versus HPV-negative TSCC ($p < 0.01$; HPV-positivity: 42.1%). HPV-positivity resulted in two up-regulated miRNAs in SLPI-positive TSCC. Of 16 further miRNAs, eight miRNAs were up- and eight were down-regulated in SLPI-negative TSCC. RT-q-PCR-validation of the four most differentially expressed miRNAs showed that miR-363 is expressed strongest in SLPI-negative/HPV-positive TSSC. *In silico*-analysis of all differentially expressed miRNAs identified miR-363, miR-210, miR-130a, and miR-181a with possible binding sites in the HPV16-E6-mRNA, but none were predicted in the SLPI-mRNA. HPV-positivity, low SLPI-levels and high miR-363-levels are significantly associated with better survival rates.

The data presented here show that miR-363 is associated with HPV-positive/SPLI-negative TSCC. The prognostic value of miR-363 suggests a role in the assumed inverse correlation of smoking and SPLI-expression in the mode of HPV-infections in tonsillar but possibly also other HNSCC.

1. Introduction

Micro (mi)RNAs are small 21–25 nucleotide long non-coding, single-stranded RNAs that are involved in e.g. signal transduction, carcinogenesis, apoptosis, and cell cycle progression. miRNAs are capable of interfering with transcription by binding to mRNAs, leading to target degradation or translational blocking. Depending on the type of mRNA they target, miRNAs may act as tumor suppressors or oncogenes [1,2]. Additionally, it is well established that viral cancers are characterized by specific miRNA patterns affecting the viral and the host genome [2]. In particular, human papillomavirus (HPV)-induced cancers like anogenital and specifically cervical cancers and a subset of head and neck squamous cells carcinoma (HNSCC) are characterized by specific miRNA-patterns [3,4]. Interestingly, the miRNA patterns of HPV-positive HNSCC and HPV-positive cervical cancer show higher similarities than the miRNA patterns of HPV-positive and HPV-negative HNSCC [4].

HNSCC are the 6th most common cancers world-wide, with approximately 600.000 new cases per year and a 5-year survival rate of approximately 50% [5]. High-risk HPV, specifically HPV16, is a causative agent for oropharyngeal SCC [6], and in particular tonsillar SCC (TSCC), the latter showing a significantly higher HPV-prevalence (30–90%) than other HNSCC, including SCC of other anatomical sites of the oropharynx [7, and references therein]. HPV-driven TSCC are increasing world-wide and represent a distinct tumor entity with identifiable epidemiologic characteristics, risk factors, and better prognosis of the patients due to better response to therapy [8].

Analyzing metastatic primary HNSCC and non-metastatic HNSCC

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showed that SLPI is the only functional transcript differentially expressed between these HNSCC subsets. This repression of SLPI was initially detected in a microarray analysis and could be confirmed on both mRNA and protein level [9]. Consecutive studies showed a significant inverse correlation of SLPI with HPV infection in HNSCC [7,10–12]. Furthermore, it was shown that tobacco smoking is associated with increasing SLPI levels in humans [11,12]. In addition, *ex vivo* data demonstrated that incubation of nasal mucosa of the lower turbinate with nicotine resulted in increased SLPI levels [12]. These observations were in agreement with the current literature on SLPI and cigarette smoke [13] and led to the suggestion that elevated expression of SLPI protects against HPV-infection [7,10–12]. This hypothesis was supported by data from a cell culture model showing that overexpression of SLPI protected HaCat cells from infection with HPV16-like particles [14]. SLPI is a 11.7-kDa nonglycosylated kazal-type serine protease inhibitor of neutrophil elastase, cathepsin G, chymotrypsin and trypsin [15]. SLPI is produced by different cell types including breast, lung, endometrium, ovary, salivary glands, and various host inflammatory and immune cells such as macrophages, neutrophils, and B lymphocytes [16–18].

In the present study one of the largest cohorts treated in a single cancer center for one specific subtype of HNSCCs, namely 126 patients with TSCC, was analyzed. The focus of the analysis was to determine if HPV-status and SLPI-expression levels affect differential expression of miRNAs. To achieve this, miRNA-arrays were performed to identify differentially expressed miRNAs dependent on SLPI-expression and HPV-status. *In silico* analysis of differentially expressed miRNAs identifies their possible binding-sites within the HPV16-E6-mRNA and the SLPI-mRNA. To evaluate potential prognostic values of SLPI and the differentially expressed miRNAs, Kaplan-Meier and Cox-regression analyses correlating these parameters and combinations thereof with HPV-status and the effect on overall survival (OS) and progression free survival (PFS) were performed.

2. Material and methods

2.1. Patients and sample preparation

FFPE samples of histophathologically confirmed palatine TSCC (n=126) were retrieved from the Institute of Pathology, Christian-Albrechts-University (CAU) Kiel, Germany. Tissue samples were obtained between 2002 and 2010 during panendoscopy or surgery (Department of Otorhinolaryngology, Head and Neck Surgery, CAU, Germany), following informed consent approved by the local Ethics Committee (D409/13). Patient characteristics were: 91 (72.2%) male; 35 (27.8%) female; aged 36.3–88.9 years (median age: 59.9 years). Patients were followed until death or up to April 2012; median follow-up: 3.18 years; range 0.12–9.29 years.

2.2. SLPI-Immunohistochemistry

Immunostaining (2 µm sections) was performed and evaluated as described, previously [9]. In brief: deparaffinized, rehydrated sections were subjected to heat-induced epitope retrieval, blocking of endogenous peroxidase and incubation in pre-immune serum, followed by incubation with a monoclonal primary antibody directed against SLPI (LifeSpan BioSciences, Seattle, WA) and incubation with a biotin-conjugated rabbit anti-mouse IgG secondary antibody (Dako, Hamburg, Germany). A peroxidase complex system (ABC-Vectorstain, Dako) was used to visualize immune reactions. Small salivary glands served as internal positive controls. Negative controls were performed by replacing primary antibody with pre-immune serum. To assess SLPI protein levels, 300 cells in at least five areas were analyzed (×400 magnification) and cases were assigned to one of the following categories (indicating the percentage of stained cells): negative: < 5%, weak: 5–30%, moderate: 31–75% and strong: > 75% of cells were stained. For statistical analysis, samples with negative and weak expression were pooled and samples with moderate and strong SLPI expression were also pooled.

2.3. Nucleic acid extraction, HPV-detection and cDNA-Synthesis

DNA was extracted from 4 to 6 consecutive 5 µm FFPE-sections (QIAamp DNA Mini Kit; Qiagen, Hilden, Germany). HPV-DNA detection, employing 50 ng of DNA per sample, was performed by PCR using the primers GP5+/GP6+ [19] and the following PCR protocol: Initial denaturation 10 min at 95 °C followed by 40 cycles of denaturation (1 min 95 °C), annealing (1 min 40 °C) and elongation (1 min 72 °C) and 5 min final elongation at 72 °C. DNA integrity was analyzed by performing PCR reactions using genomic beta-2 microglobulin (B2M) primers (Promolgene; Berlin, Germany) according to the manufacturer's protocol. Additionally, a positive control (a synthetic oligonucleotide of the HPV L1 gene, covered by the GP5+/GP6+ primers; Eurofins; Ebersberg Germany) was amplified in the GP5+/GP6+ PCRs. For RNA extraction 5 consecutive 10 µm sections were used (FFPE-RNA ready; AmpTec, Hamburg, Germany). RNA-quantity and -quality were assessed using the Nanodrop 1000 (peqlab, Erlangen, Germany) and the Tapestation 2200 (Agilent, Böblingen, Germany), respectively. RNA (200 ng) was transcribed into cDNA (TR-cDNA synthesis kit; AmpTec, Hamburg, Germany) under the following reaction conditions: 30 min at 16 °C, 30 min at 42 °C, 5 min at 85 °C followed by 5 min storage on ice. Prior to performing the miRNA-RT-qPCR assay (see 2.4), RT-qPCR using 18 S RNA primers (Promolgene; Berlin, Germany) according to the manufacturer's protocol was performed to analyze cDNA integrity.

2.4. miRNA-arrays and miRNA-RT-qPCR

Initial screening experiments were performed with a subset of TSCC samples to identify miRNAs that were differentially expressed dependent on SLPI-expression and HPV-status. miRNA-arrays were performed using 3 TSCC each, being: a) HPV-positive/SLPI-negative (< 5 cells stained positive; b) HPV-positive/SLPI-positive (> 75% cells stained positive); c) HPV-negative/SLPI-negative and d) HPV-negative/SLPI-positive. Total RNA was sent to the Biochip-Labor Universitätsklinikum Essen, Germany and analyzed on GeneChip® miRNA-arrays (Affymetrix, Santa Clara, CA). For array-expression analysis, 600 ng of total RNA were labelled with the FlashTag™ Biotin HSR RNA Labeling Kit (Affymetrix, Santa Clara, CA) and hybridized to miRNA 3.0 arrays. Washing, staining and scanning of the arrays was performed exactly as recommended by Affymetrix (Santa Clara, CA). To further confirm the miRNA-array data expression patterns of specific miRNAs were validated in all 126 tumor samples by miRNA-RT-qPCR (Applied Biosystems, Darmstadt, Germany). The array data obtained for miR-363 and miR-21 were validated in all 126 samples because these miRNAs showed the highest upregulation in HPV-positive versus HPV-negative tumors. miR-31 and miR-193b* were validated because they showed the strongest down-regulation in HPV-positive versus HPV-negative tumors. In addition these 4 miRNAs exhibited the same regulation pattern in a previously performed miRNA array study [4]. Further validation experiments were performed for miR-210, miR-130a, and miR-181a. *In silico* analysis (see 2.5) predicted that these miRNAs, like miR-363, might have possible binding-sites in the HPV16-E6-mRNA, but no predicted binding sites in the SLPI-mRNA, and might therefore play a role in the previously reported significant inverse correlation between SLPI-expression and HPV-infection in HNSCC [7,10–12]. For normalization, miR-191 and miR-RNU44 were used, neither of which were differentially expressed in HPV-positive and HPV-negative TSCC [4]. PCR conditions for all miRNA-RT-qPCR assays were: 10 min initial denaturation (95 °C); 40 cycles of denaturation (95 °C; 15 s) followed by annealing / extension (60 °C, 60 s).

2.5. In silico analysis of miRNA/mRNA interactions

To analyze possible interactions of differentially expressed miRNAs with SLPI- and HPV16-E6-mRNA, the following algorithms were used: Segal Lab online miRNA prediction tool (https://132.77.150.113/pubs/mir07/mir07_prediction.html), DIANAmT (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index) and Targetscan 6.2 (http://www.targetscan.org/) [20–22]. The mRNA sequences of the SLPI-mRNA (accession number NM_003064.3) and the HPV16-E6-mRNA (accession number NC_001526.4) were entered and the interaction-prediction tools were run at default settings. Possible miRNA/mRNA interactions were accepted if two out of three algorithms indicated a match.

2.6. Statistical analysis

qPCR data were analyzed by the ΔΔCt method [23] using the mean Ct-values obtained for miR-191 and miR-RNU44 for normalization purposes. For mathematical details, please see figure legends. The median ΔΔCt value was used to determine the high versus low cut-off for Kaplan-Meier analysis, Cox regression analysis and Fisher's exact testing. For Kaplan-Meier analysis, statistical end points were PFS and OS, defined as time from diagnosis to progression and last follow-up or death, respectively. Factors tested included HPV-status, as well as SLPI- and miRNA-expression. Log rank test (SPSS 20.0 software) was used to detect significant differences. In addition, Cox multivariate analysis (forward stepwise, SPSS 20.0 software) was performed to adjust for potential confounding factors. To this end TNM staging, UICC, age (cut-off median age of the patient population), sex and smoking habit (smoker versus nonsmoker was used as cut-off) were included in the model. For T stage, T1 and T2 samples were pooled, as were T3 and T4 samples. For N stage, N0 samples were tested versus N1-2a and > N2b, respectively. Fisher's exact test correlated miRNA-, SLPI-expression and HPV-status (SPSS 20.0 Software). p-values < 0.05 were considered statistically significant. Statistical analysis of the miRNA array data was performed by Signal summarization (RMA algorithm), followed by ANOVA testing using PartekGS (Partek Inc., St. Louis, MO). Microarray data were considered significant when differences were 2-fold higher or lower and p-values were < 0.01.

3. Results

3.1. HPV-status and SLPI-expression

HPV-DNA analysis was performed in all 126 TSCC samples; HPV-positive: 42.1%; n=53 [age: 63.6 ± 7.9 years (range 47.0–88.9 years)]. HPV-negative patients were 57.6 ± 9.0 years old; (range 36.3–78.4 years; p=0.0002).

SLPI-expression was as follows: of 126 specimens 9 (7.1%) were SLPI-negative, 32 (25.4%) showed weak, 55 (43.7%) moderate, and 30 (23.8%) strong SLPI-expression.

Correlating HPV-status and SLPI-expression: out of 53 HPV-positive tumors, 6 (11.3%) were SLPI-negative, 12 (22.6%) showed weak, 22 (41.5%) moderate, and 13 (24.5%) strong SLPI-expression. Out of 73 HPV-negative tumors, 3 (4.1%) were SLPI-negative, 20 (27.4%) showed weak, 33 (45.2%) moderate, and 17 (23.3%) strong SLPI-expression; p=0.460.

3.2. miRNA RT-q-PCR and in silico analysis

Within the subset of HPV-positive tumor samples, miR-363-expression was increased by 14.25-fold, miR-21 showed a 7.77-fold increase, miR-31 expression was downregulated by 5.21-fold and miR-193b* exhibited a 5.75-fold down-regulation, which is in line with previously observed results [4] (Fig. 1). Further analysis of these miRNAs showed the highest expression of miR-363 in SLPI-negative/HPV-positive tumors, with significant differences observed only between SLPI-negative/HPV-positive and SLPI-negative/HPV-negative tumors (Fig. 2).

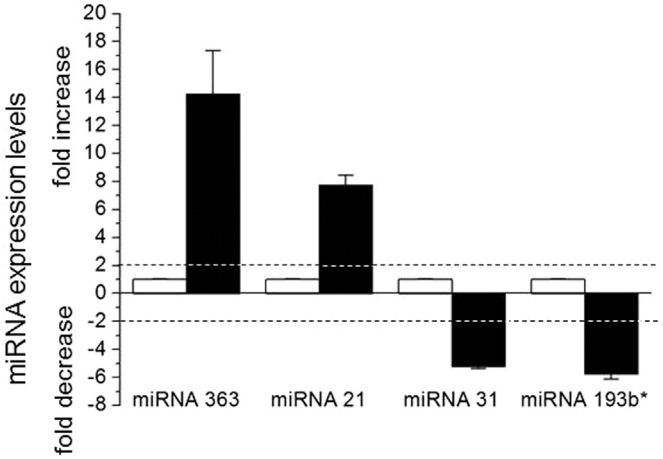

Fig. 1. Expression of miRs-363, −21, −31 and −193b* in TSCC. miRNA-expression in all 126 TSCC dependent on HPV-status of the tumor are shown. ΔCt values obtained in HPV-negative tissue samples (with bars) were set as "1" and fold changes of HPV-positive samples (black bars) were calculated according to Pfaffl [23] as $1/2^{\wedge\Delta CtHPVnegative - \Delta CtHPVpositive}$ in case of decreased gene expression and as $2^{\wedge\Delta CtHPVnegative - \Delta CtHPVpositive}$ in case of increased gene expression. Dotted lines indicate 2-fold changes of gene expression (both decrease and increase) which are considered statistically significant [23].

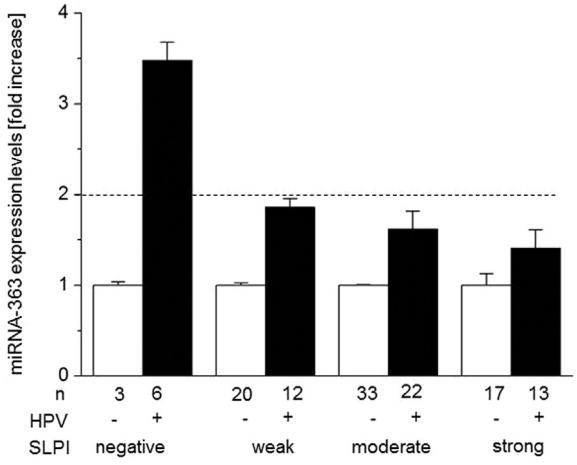

Fig. 2. Expression of miR-363 in HPV-positive and HPV-negative TSCC dependent on SLPI-expression. Expression of miR-363 in all 126 TSCC dependent on HPV-status and SLPI-expression of the tumor are shown. Significant differences in miR-363-expression between HPV-positive and HPV-negative tumors were only seen in SLPI-negative tumors. ΔCt values obtained in HPV-negative tissue samples (white bars) were set as "1" and fold changes of HPV-positive samples (black bars) were calculated according to Pfaffl [23] as $2^{\wedge\Delta CtHPVnegative - \Delta CtHPVpositive}$. The dotted line indicates 2-fold changes of gene expression which is considered statistically significant [23].

In silico screening revealed that all previously differentially expressed miRNAs [4] had possible binding-sites in the HPV16-E6- and the SLPI- mRNA. Only miR-363 had no predicted binding-sites in the SLPI-mRNA but possible binding sites in the HPV16-E6-mRNA are described.

3.3. miRNA-array study

Based on the differential expression of miR-363 in SLPI-negative/HPV-positive tumors and the lack of possible miR-363 binding-sites in the SLPI-mRNA, miRNA-arrays were performed to possibly identify further miRNAs differentially expressed dependent on SLPI-expression and HPV-status. Overall, 24 miRNAs were up-regulated in HPV-

Table 1
Fold change levels of miRNAs expressed differently in HPV-positive versus HPV-negative TSCC.
Table 1 shows the results of the miRNA arrays performed on three TSCC, each, of the following groups: HPV-positive/SLPI-negative (< 5% cells stained positive); HPV-positive/SLPI-positive; HPV-negative/SLPI-negative and HPV-negative/SLPI-positive (< 75% cells stained positive), when accepting fold changes > −2 and < 2 and p < 0.01 as statistically significant. In addition, all differentially expressed miRNAs were checked for potential binding sites in the SLPI- and HPV16-E6-mRNA and we analyzed whether these miRNAs have previously been reported to play a role in HNSCC and cervical cancers. Furthermore, target analysis was performed for the genes differentially expressed in HNSCC.

Probe set ID	p-value	fold change HPV+/ HPV-	possible mRNA binding site		HNSCC related	cervix related	main targets in HNSCC
			SLPI	HPV 16 E6			
miR−363 A	0,000340	13,22	no	yes	[2,4,24–26]	[27]	Myo−1B [2], podoplanin [24]
miR−21C	0,000824	5,10	yes	yes	[4]	[28,29]	
miR−378c C	0,002687	4,88	yes	yes	[25]		
miR−378i C	0,002892	4,23	yes	yes			
miR−210 A	0,003616	3,52	no	yes	[2,30]	[31]	hypoxia-inducible factor 1; carbonic anhydrase 9 [30]
miR−378f C	0,0012967	3,49	yes	yes			
miR−422a A	0,0015703	3,23	yes	yes		[29]	
miR−150 A	0,0034886	3,13	yes	yes	[4,25,32]		MYB; NFIB [32]
miR−15a C	0,005975	3,10	yes	yes	[4,32]	[28]	MYB; NFIB [32]
miR−378C	0,001662	3,03	yes	yes		[29]	
miR−182 A	0,0045582	3,01	yes	yes		[29]	
miR−200a C	0,001974	2,97	yes	yes		[28]	
miR−20b C	0,0039406	2,90	yes	yes	[4,25]	[29]	
miR−34a B	0,001689	2,82	yes	yes	[26]	[31]	
miR−34c−5p A	9,81E−05	2,65	yes	yes			
miR−141C	0,001824	2,54	yes	yes		[28]	
miR−130a A	0,0015385	2,52	no	yes			
miR−378d C	0,0010206	2,46	yes	yes			
miR−3188 A	0,0033705	2,36	no	no			
miR−155C	0,0011095	2,36	yes	yes	[2,4,33]	[34]	TP53-miRNA pathways [33]
miR−378g C	0,0027823	2,26	yes	yes			
miR−30a C	0,0042704	2,16	yes	yes	[35]		
miR−27b A	0,007450	2,01	yes	yes	[36]	[37]	hepatic growth factor [36]
miR−378* B	0,0035958	2,01	yes	yes			
miR-3126−3p A	0,0015451	−2,04	no	no			
miR−1275 A	0,0011058	−2,06	yes	yes			
miR-4749−3p A	0,0038285	−2,10	no	no			
miR−181a A	0,002073	−2,12	no	yes	[26]	[28]	
miR−181b A	0,0033700	−2,12	yes	yes			
miR-486−3p A	9,98E−06	−2,18	yes	yes			
miR−1976 A	0,0049814	−2,35	no	no			
miR-342−5p A	0,0044567	−2,93	yes	yes			
miR−31C	0,002132	−4,95	yes	yes	[4]	[38]	
miR−193b* C	0,0049814	−4,99	yes	yes	[2,4,25,39]	[40]	neurofibromin [39]

A indicates miRNAs exclusively regulated in SLPI-negative TSCC; B those exclusively regulated in SLPI-positive TSCC, and C indicates miRNAs regulated independent of the SLPI-status.

positive tumors when compared to HPV-negative samples, with miR-363 showing the highest up-regulation (13.22-fold). Ten miRNAs were down-regulated in HPV-positive tumors when compared to HPV-negative tumors. Dependent on SLPI-expression 17 miRNAs were differentially expressed in SLPI-negative tumors and 2 were differentially expressed in SLPI-positive TSCC. The remaining 15 miRNAs were regulated independent of SLPI-expression (Table 1). Similarly to previously published results [4], miR-363 and miR-21 were found to be the most up-regulated, and miR-31 and miR-193b* were the most down-regulated miRNAs. Several other miRNAs were regulated in a similar manner as described, elsewhere [4], but additional differentially regulated miRNAs were identified (Table 1).

Moreover, three more miRNAs with possible binding-sites in the HPV16-E6-mRNA but no predicted binding-site in the SLPI-mRNA were found (i.e. miRs-210 and 130a showing a 3.52- and 2.52-fold increase in HPV-positive versus HPV-negative TSSC, respectively, and miR-181a showing a 2.12-fold decrease in HPV-positive versus HPV-negative TSSC).

3.4. Database search identifying possible roles for the differentially expressed miRNAs in cervical and head and neck cancers and HNSCC-related target genes

Among the 34 identified differentially expressed miRNAs, 16 (47%) were previously described in HNSCC. Since it was previously shown that the miRNA pattern of HPV-positive TSCC and HPV-positive cervical cancer specimens showed higher similarities than HPV-positive and HPV-negative TSCC samples [4], a literature search was performed to establish if the differentially regulated miRNAs detected in our study had been mentioned before in the context of either cervical cancer or tobacco induced (mostly HPV-negative) TSCC. Of the 34 differentially expressed miRNAs, 11 (32%) were also described as differentially regulated in cervical cancer. Only miR-30a was formerly mentioned in the context of tobacco-induced HNSCC. Target genes for 7 (44%) of the 16 miRNAs reported in HNSCC are described as (i) transcription factors, (ii) genes involved in cancerogenesis, or (iii) tumor markers [Table 1; PubMed (http://www.ncbi.nlm.nih.gov/pubmed) results November 2016].

3.5. Kaplan-Meier analysis of HPV-status, SLPI- and miRNA-expression

Patients with HPV-positive TSSC showed better OS (p=0.025) and PFS (p=0.015; Fig. 3A, B). To analyze SLPI-expression, negative/weak and moderate/strong cases were pooled, demonstrating survival advantages for patients with SLPI-negative/weak tumors; OS (p=0.029) and PFS (p=0.049; Fig. 3C, D). HPV-negativity in combination with moderate/strong SLPI-expression resulted in the worst outcome; OS

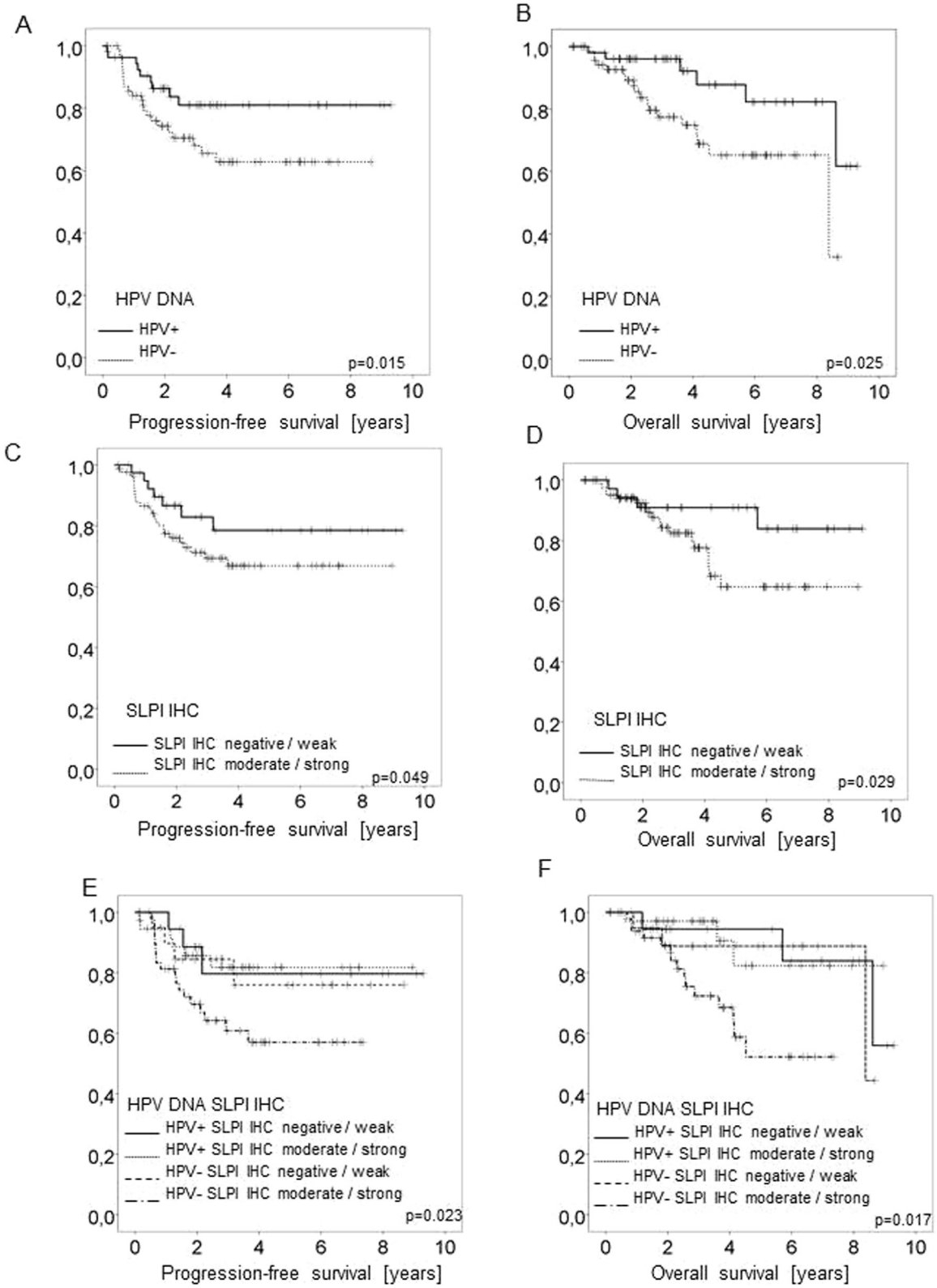

Fig. 3. Kaplan-Meier curves showing overall survival (right panel) and progression-free survival (left panel), respectively, for HPV-DNA (A, B), SLPI-expression (C, D), and the combined effect of HPV-DNA and SLPI-expression on overall survival (F) and progression-free survival (E). The data show that HPV-positivity as well as negative/weak SLPI-expression provide a survival advantage for the patients. The predictive factor for overall and progression-free survival appears to be even better when combining these 2 parameters indicating that HPV-negativity together with moderate to strong SLPI-expression is the worst prognostic predictor.

(p=0.017) and PFS (p=0.023; Fig. 3E, F).

Expression of miRs −21, −31, −193b*, −210, 130a, and −181a had no prognostic value. High miR-363-expression was correlated with better OS (p=0.013) and PFS (p=0.022; Fig. 4A; B). Low miR-363 and moderate/strong SLPI-expression showed the worst outcome (p=0.021 for OS; $p > 0.05$ for PFS; Fig. 4C; D); similarly HPV-negativity in combination with low miR-363-expression had the worst outcome (PFS > 0.05; OS p=0.048; Fig. 4E; F).

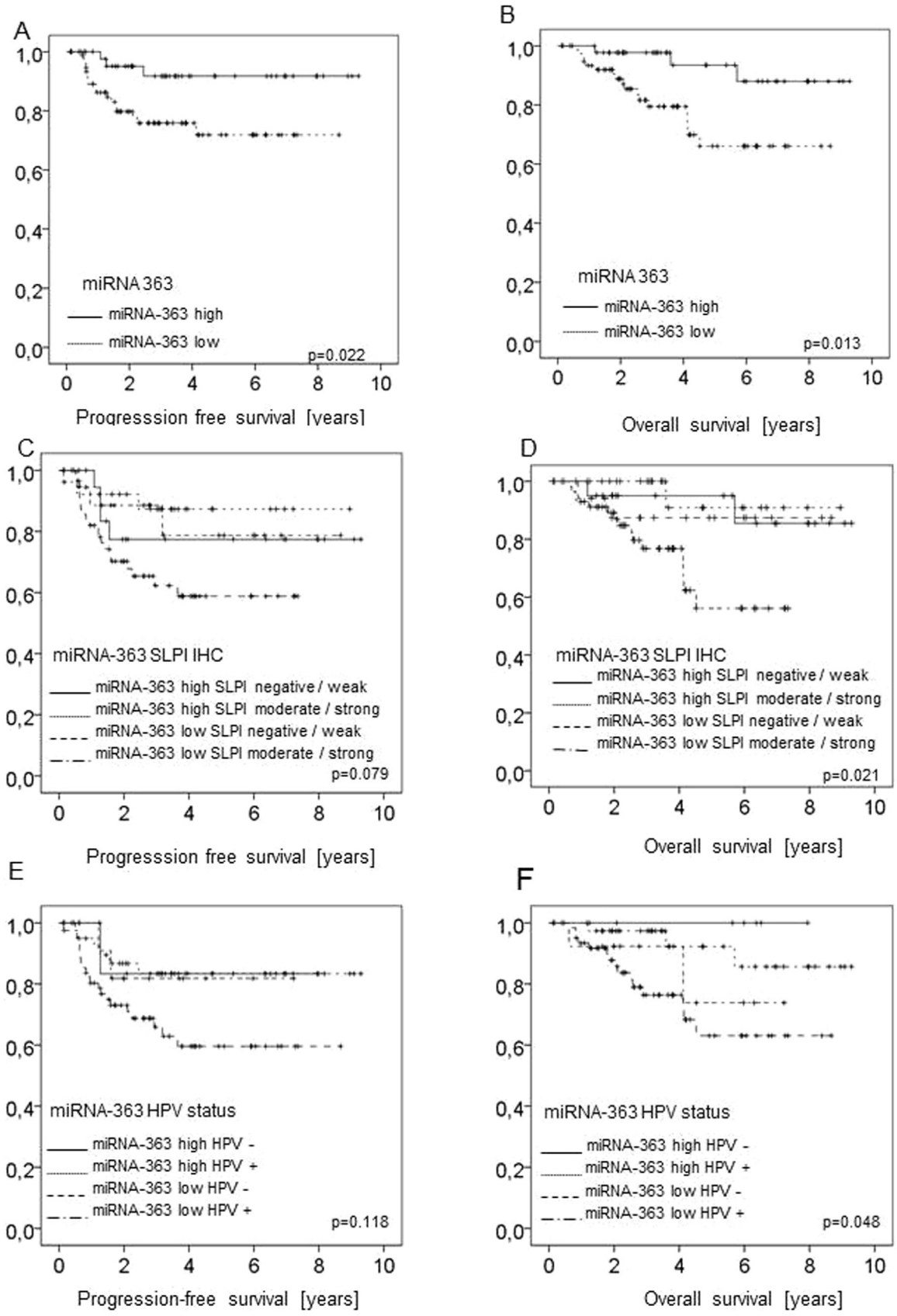

Fig. 4. Kaplan-Meier curves showing overall survival (right panel) and progression-free survival (left panel), respectively, for miR-363-expression (A, B), for miR-363-expression in combination with SLPI-expression (C, D), and in combination with HPV-status on overall survival (F) and progression-free survival (E). The data show that high expression of miR-363 was correlated with better overall and progression-free survival. The combination of miR-363-expression with SLPI-expression showed that patients with low miR-363- and moderate/strong SLPI-expression had the worst outcome. Combining miR-363-expression with HPV-status revealed that patients with low miR-363-expression and HPV-negative tumors had the worst outcome with, however, only overall survival showing a significant correlation.

Table 2
Cox multi-variant regression analysis.

The upper panel shows the result for OS the lower for PFS applying Cox multi-variant regression (forward stepwise) including the shown parameters into the model. In addition the results for the log rank test following Kaplan-Meier analysis are given. M0, HPV-positivity, high miR-363 levels, negative/weak SLPI expression and non-smoking are associated with better OS and PFS.

OS				
	Hazard Ratio	95.0% CI	p-value	p (log-rank)
M-stage	4.225	(2.222 – 8.032)	< 0.001	< 0.001
miR–363	0.301	(0.094 – 0.966)	0.013	0.013
HPV	0.481	(0.238 – 0.972)	0.042	0.025
SLPI	2.023	(1000 – 4.092)	0.049	0.029
smoking	2.602	(1.015 – 6.669)	0.046	0.038
UICC	1.087	(0.832 – 1.422)	0.541	0.541
T-stage	1.092	(0.454 – 1.892)	0.835	0.798
N-stage	1.366	(0.908 – 2.055)	0.135	0.105
sex	0.544	(0227 – 1.301)	0.171	0.165
age	1.570	(1.299 – 2.088)	0.088	0.084
PFS				
	Hazard Ratio	95.0% CI	p-value	p (log-rank)
M-stage	4.681	(2.215 – 8.531)	< 0.001	< 0.001
miR–363	0.421	(0.190 – 0.931)	0.033	0.022
HPV	0.484	(0.230 – 1.015)	0.031	0.015
SLPI	1.994	(0.950 – 4.186)	0.068	0.049
smoking	4.320	(1.361 – 13.709)	0.044	0.032
UICC	1.884	(0.682 – 3.145)	0.351	0.249
N-stage	1.050	(0.656 – 1.681)	0.837	0.579
T-stage	1.246	(0.546 – 2.846)	0.602	0.581
sex	0.572	(0.272 – 1201)	0.140	0.135
age	1.624	(1.259 – 2.505)	0.294	0.203

3.6. Cox regression analysis

In a Cox multivariate analysis (forward stepwise) TNM staging, UICC, age, sex, smoking habit, HPV-status, SLPI- and miR-353 expression were included in the model. As shown in Table 2, apart from HPV-status, miR-363- and SLPI-expression, only M stage and smoking habit had additional effect on OS and PFS.

3.7. Correlating miR-363 with SLPI-expression, HPV-status and smoking habit

Only miR-363 was correlated with SLPI-expression, HPV-status, and smoking habit, because of miR-363 (i) singularly showing prognostic value, (ii) being the only miRNA with altered expression in the entire cohort, and (iii) having no predicted binding-site in the SLPI-mRNA. Of 39 patients with negative/weak SLPI-expression, 11 (28.2%) showed low and 28 (71.8%) high miR-363-expression. Of 87 samples with moderate/strong SLPI-expression, 60 (69.0%) had low and 27 (31.0%) high miR-363-expression ($p < 0.0001$). HPV-negativity (n=73) was associated with low miR-363-expression in 63 (86.3%) cases and high miR-363-expression in 10 (13.7%) cases. HPV-positivity (n=53) was linked to low expression in 8 (15.1%) cases and to high miR-363-expression in 45 (84.9%) cases ($p < 0.0001$). Correlating miR-363-expression with smoking habit demonstrated that of 32 non-smokers, 11 (34.4%) showed low and 21 (65.6%) high miR-363-expression. Of 94 patients with smoking habit, (61 (64.9%) showed low and 33 (35.1%) high miR-363-expression (p=0.004).

4. Discussion

The data presented describe an involvement of miR-363 in the hypothesized causative association between SLPI and HPV-infection in HNSCC, thereby providing further evidence that SLPI-expression and HPV-status in TSCC and possibly other HPV-associated cancers are inversely correlated.

We show that miR-363 was highly up-regulated in HPV-positive TSSC. In accordance with a previous report [4], we detected that miR-363 was up-regulated approximately twice as much than the next strongest up-regulated miRNA. We therefore questioned whether this difference might be related to the shown inverse correlation between SLPI-expression and HPV-status in HNSCC [7,11,12]. To test this hypothesis, miR-363-expression was stratified for the combination of SLPI-expression and HPV-status. This analysis showed that significant increases of miR-363 expression were only seen when comparing SLPI-negative/HPV-positive versus SLPI-negative/HPV-negative tumors. Out of the four miRNAs with no potential binding site in the SLPI mRNA, only miR-363 was confirmed in the entire cohort of 126 TSCC as differentially regulated in regard to the HPV-status of the patients, confirming the possible relevance of miR-363. Furthermore, out of the tested miRNAs, only miR-363 was of any prognostic value. In agreement with the current literature [2] high miR-363 expression levels correlated with better OS and PFS. Correlating HPV-status and miR-363 demonstrated that patients with low miR-363-levels and HPV-negative tumors had the worst outcome. This is not surprising, considering the previously demonstrated inverse correlation between SLPI-expression and HPV-status, and given the additional finding that low miR-363-levels and moderate/strong SLPI-levels were also associated with least favorable OS. Survival disadvantages associated with low miR-363-levels and HPV-negative tumors were also seen in cervical cancer patients [27]. These results might be explained by the in vitro finding that the HPV-16 E6 oncoprotein stimulates miR-363-expression [41]. In this context it is, however, surprising that the group of patients presented here with high miR-363 levels, yet HPV-negative tumors showed 100% OS. The reason for this remains unclear, but might be explained by the relatively small number of patients (n=10). Positive HPV-status of HNSCCs and particularly in TSCC is linked to better OS and PFS [42]. In agreement with this finding the patients analyzed here with HPV-positive TSCC showed better OS and PFS. The excellent survival data of the HPV-positive patients investigated here is comparable to other studies [43 and references therein]. All of the patients included in this study were treated with surgery and selective neck dissection (94%), followed by adjuvant radio(chemo)therapy, if indicated (68%). The excellent survival data of HPV-positive patients is a result of this treatment, avoiding toxicity and morbidity of high dose chemotherapy in the treatment regime of primary radio(chemo)therapy. In this context it might be of note that in a previous study [44] analyzing survival data of TSCC-patients, unlike in the cohort analyzed here, UICC, T- and N-stage of the tumors and age of the patients, were found to be confounding factors of OS when using a Cox regression analysis. The data presented here reveal only smoking habit and M-stage in addition to HPV-status and SLPI- and miR-363-expresion as confounding factors for OS and PFS.

Not surprising, given our hypothesis regarding the correlation between SLPI-expression and HPV-infection, patients with negative/weak SLPI-expression showed significantly better OS and PFS. HPV-negativity in combination with moderate/strong SLPI-expression was the worst predictor for OS and PFS (Fig. 2D; E). Recently, a prognostic value for SLPI was shown for oral SCC [45] and ovarian cancer [46]. Noorlag and co-workers [45] associated higher SLPI-expression with better survival. The difference to our data might be attributed to the low HPV-prevalence (< 1%) in oral SCC [45]. Overall the survival data presented here strengthens the hypothesis postulating an inverse correlation between SPLI-expression and HPV-status, with miR-363 having a modulatory effect, possibly not only in tonsillar, but also in other HPV-associated cancers.

The microRNA-array data presented here show that several miRNAs are differentially expressed dependent on SLPI-expression and HPV-status. Approximately 50% of the differentially expressed miRNAs detected here were previously described in HNSCC (Table 1). Of these miRNAs, some were previously also identified in cervical cancer (Table 1). Only miR-30a was previously found up-regulated in alcohol-induced (mostly HPV-negative) HNSCC [35] further implying that HPV-positive and HPV-negative TSCC are separate entities

characterized by different miRNA-profiles.

The differentially expressed miRNAs mostly target transcription factors, hence, participate in the altered gene expression of tumors. Focusing on targets for miR-363 specifically in HNSCC, it was previously shown that miR-363 targets myosin-1B [2] and podoplanin [24]. Interestingly, increased miR-363 levels led to down-regulation of both podoplanin and myosin-1B. Resulting in impaired migratory activity of HNSCC cells and reduced metastases in HPV-positive HNSCC [2,24,47]. However, metastatic HNSCC are characterized by e.g. low miR-363 and high podoplanin levels [2] while it could be shown that SLPI is reduced in these tumors [9]. Whether the fact that miR-363 has no potential binding site in the SLPI-mRNA presents a possible explanation for the simultaneous expression of miR-363 and SLPI in metastatic HNSCCs remains to be determined. But due to the lack of a potential binding site of miR-363 in the SLPI mRNA, a direct down-regulation of SLPI through increased miR-363 levels in HPV-positive HNSSC can be excluded.

Surprisingly, the data presented here only show a trend towards the previously reported inverse correlation between SLPI-expression and HPV-status [7,11,12]. This lack of significance might be attributed to the fact that 31.2% of the HPV-positive patients were smokers, compared to only 15.7% in a cohort previously analyzed by us, comprising 205 HNSCC patients [7]. Our previous results suggested that smoking might be one possible factor leading to the inverse correlation between HPV-status and SLPI-expression. This assumption is based on the finding that *ex vivo* nicotine incubation of nasal mucosa resulted in increased SLPI-expression [12]. The possible role of miR-363 in the inverse correlation between HPV-status and SLPI-expression is further supported by the finding that high miR-363-levels are associated with HPV-positive TSCC with low SLPI-expression; furthermore, high miR-363-expression is mostly found in non-smokers supposed to develop HPV-positive HNSCC [8].

5. Conclusion

The data presented here provide further evidence that SLPI plays a pivotal role in developing HPV-driven HNSCC, and possibly other HPV-associated cancers. SLPI-expression is correlated with HPV-status, resulting in the differential regulation of miR363 in HPV-positive/SPLI-negative TSCC samples. These observations, together with the prognostic value of miR-363 suggest a role for this miRNA in the proposed SLPI-modulated HPV-infection of tonsillar, but possibly also other HNSCC, cervical, and anogenital cancers.

The so far unidentified underlying mechanism and the validation of the assumption that SLPI-, miR-363-expression and HPV-status are linked in other HPV-associated cancers warrant future research.

Conflict of interests

None

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