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A let-7 microRNA polymorphism in the *KRAS* 3′-UTR is prognostic in oropharyngeal cancer

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ABSTRACT

Introduction: This study aimed to investigate the effect of genetic polymorphisms in miRNA sequences, miRNA target genes and miRNA processing genes as additional biomarkers to HPV for prognosis in oropharyngeal squamous cell carcinoma (OPSCC) patients. Secondarily, the prevalence of HPV-associated OPSCC in a European cohort was mapped.
Methods: OPSCC patients (n = 122) were genotyped for ten genetic polymorphisms in pre-miRNAs (*pre-mir-146a*, *pre-mir-196a2*), in miRNA biosynthesis genes (*Drosha*, *XPO5*) and in miRNA target genes (*KRAS*, *SMC1B*). HPV status was assessed by p16 immunohistochemistry (IHC) and high-risk HPV in situ hybridization (ISH) or by p16 IHC and PCR followed by enzyme-immunoassay (EIA). Overall and disease specific survival were analysed using Kaplan–Meier plots (log-rank test). Cox proportional hazard model was used to calculate hazard ratios (HR).
Results: The overall HPV prevalence rate in our Belgian/Dutch cohort was 27.9%. Patients with HPV$^+$ tumours had a better 5-years overall survival (78% *vs.* 46%, p = 0.001) and a better 5-years disease specific survival (90% *vs.* 70%, p = 0.016) compared to patients with HPV$^-$ tumours. In multivariate Cox analysis including clinical, treatment and genetic parameters, HPV negativity (HR = 3.89, p = 0.005), advanced T-stage (HR = 1.81, p = 0.050), advanced N-stage (HR = 5.86, p = 0.001) and >10 pack-years of smoking (HR = 3.45, p = 0.012) were significantly associated with reduced overall survival. The variant G-allele of the *KRAS-LCS6* polymorphism was significantly associated with a better overall survival (HR = 0.40, p = 0.031).
Conclusions: Our results demonstrate that OPSCC patients with the *KRAS-LCS6* variant have a better outcome and suggest that this variant may be used as a prognostic biomarker for OPSCC.

1. Introduction

The increase in incidence of oropharyngeal squamous cell carcinoma (OPSCC) over the last 20 years has mainly been attributed to human papillomavirus (HPV) infection [1–3]. Depending on geographical location and time period studied, HPV prevalence rates in oropharyngeal cancer (OPC) range from 23% to 73% [4]. Among HPV-positive OPC, HPV16 is the predominant genotype, accounting for approximately 95% of cases. HPV-associated OPSCC seems to be a distinct clinical entity with a better prognosis than HPV-negative tumours [5]. The strong prognostic effect of HPV status can be improved by combining it

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with smoking status and tumour and nodal stage [6,7]. Refining patient stratification by considering additional biomarkers next to HPV status and clinical parameters, may ensure appropriate therapy and therefore better survival and improved quality of life.

MicroRNAs (miRNAs) are endogenous short non-coding RNA molecules that regulate gene expression by binding to the 3′-untranslated region (UTR) of protein coding transcripts, in turn triggering mRNA degradation or translational repression [8]. The biogenesis of miRNAs is a complex process involving multiple proteins. First, large primary precursors of miRNAs (pri-miRNA) are transcribed by RNA polymerase II and cleaved to miRNA precursor (pre-miRNAs) by the nuclear complex containing the RNASEN (Drosha) and DGCR8 proteins [9]. Then, the pre-miRNA is translocated to the cytoplasm through the assistance of RAN-GTPase and Exportin-5 (XPO5), where it is further processed by a protein complex including DICER, leading to the production of double-stranded miRNA duplex. Subsequently, one strand of miRNA is incorporated into RNA-induced silencing complex (RISC) including GEMIN3 and GEMIN4, which mediates expression of target genes [10]. It was estimated that a single miRNA can target hundreds of mRNAs and nearly 30% of protein-coding genes in the human genome can be regulated by miRNAs [11]. MiRNAs are involved in important biological processes related to apoptosis, proliferation, differentiation, angiogenesis and immune response [12]. Furthermore, miRNA expression alterations have been linked to initiation, progression, metastasis and treatment outcome of human cancers, including OPSCC [13,14].

Genetic polymorphisms within miRNA sequences, target genes or processing genes are thought to have a functional impact on how miRNAs are active in tumours. The variant allele of the rs61764370 polymorphism in a let-7 miRNA complementary site (LCS6) of the *KRAS* 3′-UTR (*KRAS-LCS6*) has been shown to affect the survival of oral cancer patients [15]. The rs11614913 polymorphism of *pre-mir-196a2* has been shown to enhance the survival of pharyngeal, oropharyngeal and oral cancer patients [16–18], and the *pre-mir-146a* rs2910164 polymorphism was shown to be prognostic for oropharyngeal cancer [17]. Several genetic variations in miRNA processing and target genes were found to be associated with the risk of second primary tumour and/or recurrence in patients with early stage head and neck cancer. The most significant variation, rs3747238, was located in a miRNA binding site of the *SMC1B* gene [19].

This study aimed to determine the prevalence of HPV-associated OPSCC in a European cohort and to investigate the effect of genetic polymorphisms in miRNA sequences, target genes and processing genes as additional markers to HPV for prognosis in OPSCC patients.

2. Materials and methods

2.1. Study population

The study population consisted out of 122 OPSCC patients treated between April 2003 and May 2012. Only patients receiving curative radiotherapy and from whom biological material was available, were included. A total of 57 patients were selected from the MAASTRO Clinic blood biobank database (The Netherlands) which prospectively collects blood samples of all patients scheduled for curative radiotherapy. Sixty-five patients treated at the Ghent University Hospital (Belgium) and participating in radiogenomics studies were also selected. The study was approved by the ethical committees of both centres and all study participants provided informed consent. The MAASTRO Clinic cohort study was filed at clinicaltrials.gov (no. NCT01084785).

Clinical, treatment and follow-up data were available in research databases. For every patient who was alive at the point of analysis but had not been seen in the clinic for the preceding 12 months, the general practitioner was contacted to ensure that the patient was still alive. Patients were classified as died by disease only if they had documented progressive or recurrent cancer. Clinical data and treatment details are presented in Table 1. The majority of the patients were treated with intensity-modulated radiotherapy (IMRT) opposed to three-dimensional conformal radiotherapy (3DCRT) and computer-planned conventional radiotherapy with opposing latero-lateral fields and an anterior lower-neck field. The median total tumour dose was 69 Gy at 2.0–2.70 Gy/fraction (1 patient received 1.66 Gy/fraction). The details of the different radiotherapy treatment regimens can be found in the online supplement A. Forty-seven percent of the patients received platinum-based chemoradiotherapy. Eight patients (14%) received daily low dose cisplatinum at 6 mg/m^2 before each radiotherapy fraction. Four patients (7%) were treated with weekly cisplatinum at 30 mg/m^2. For 45 patients (79%), cisplatinum at 100 mg/m^2 was planned on days 1, 22 and 43. Due to toxicity, 15 patients only received two courses and 4 patients only received one course of cisplatinum.

2.2. HPV detection

HPV status was determined on formalin-fixed paraffin-embedded (FFPE) biopsy and resection material of OPSCC retrieved from the archives of the departments of pathology of both participating university hospitals. For the Gent patients, HPV infection was assessed by p16 immunohistochemistry (IHC) and high-risk HPV in situ hybridization (ISH). For the Maastricht patients, HPV infection was determined by p16 IHC and p16 positive samples were retested by PCR followed by enzyme-immunoassay (EIA).

2.2.1. p16 immunohistochemistry

p16 IHC was carried out on 1.5 μm thick tissue sections using the CINtec® p16 Histology (Ventana Medical Systems) on a BenchMark XT automated stainer (Ventana Medical Systems) under ISO15189:2007 (Gent) or CCKL (Maastricht) accreditation. p16 IHC was scored as positive if there was strong and diffuse nuclear and cytoplasmic staining present in more than 70% of tumour cells. All other staining patterns were scored as negative.

2.2.2. GP5+/6+ PCR enzyme immunoassay

Genomic DNA was extracted from 30 μm thick tissue sections using the FFPE kit of Gentra Puregene (Qiagen, The Netherlands). HPV specific PCR with GP5+/6+ primers was performed. For samples without amplification, Specimen Control Size ladder was used for DNA quality control. HPV-typing was performed by EIA using a single probe for HPV type-16 and cocktails of probes for 14 high-risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) and 4 low-risk (6, 11, 40, 42) HPV types. Assays were run in triplicate, with positive and negative controls.

2.2.3. High-risk HPV in situ hybridization

High-risk HPV ISH was performed on 2–3 μm thick tissue sections using the Inform HPV III Family B probe (Ventana Medical Systems) on a BenchMark XT automated stainer (Ventana Medical Systems) under ISO15189:2007 accreditation. The probe cocktail detects HPV types 16,18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 66. The high-risk HPV ISH test was scored by a pathologist and was considered positive if a discrete, blue coloured, precipitated reaction product within the tumour cells was observed.

2.3. Genotyping

Two polymorphisms in pre-miRNAs (*pre-mir-146a* rs2910164, *pre-mir-196a2* rs11614913), six polymorphisms in miRNA biosyn-

Table 1
Characteristics of the study population in relation to outcome.

	n	%	Overall survival	
	122		Deaths	% surviving 5 years[a]
Gender				
Men	99	81.1	50	52.8
Woman	23	18.9	9	64.1
Age (years)				
Median	60.5		–	–
Range	37.2–77.9		–	–
Nicotine use				
Never	12	10.1	2	80.2
Ever	107	89.9	56	51.3
Missing	3			
Pack-years				
≤10	23	21.1	5	73.2
>10	86	78.9	50	47.7
Missing	13			
Alcohol use				
Never	11	9.5	4	52.6
Ever	105	90.5	52	55.4
Missing	6			
Drinks/week				
<10	44	37.3	13	69.1
≥10	74	62.7	44	46.6
Missing	4			
Subsite tumour				
Tonsil	55	45.1	25	58.3
Other	67	54.9	34	51.4
T-stage				
1–2	67	54.9	25	68.5
3–4	55	45.1	34	38.7
N-stage				
0–1–2a	45	37.5	14	76.2
2b–2c–3	75	62.5	44	39.6
Missing	*2*			
Tumour HPV status				
Positive	34	27.9	6	78.0
Negative	88	72.1	53	45.9
Treatment				
RT[b]	48	39.3	21	59.7
RT[b] + ChT[c]	49	40.2	26	43.5
Surgery + RT[b]	17	13.9	9	70.6
Surgery + RT[b] + ChT[c]	8	6.6	3	48.6
Radiotherapy type				
Non-IMRT	37	30.3	18	64.9
IMRT	85	69.7	41	48.5
Total tumour dose (Gy)				
Median	69.0		–	–
Range	66.0–81.0		–	–
Dose/fraction (Gy)				
Median	2.0		–	–
Range	1.66–2.70		–	–
Fractionation scheme				
Daily	93	76.2	47	50.9
Hybrid[d]	29	23.8	12	64.8
Treatment time[e] (days)				
Median	44.0		–	–
Range	28.0–59.0		–	–
Centre				
Gent	65	53.3	36	47.2
Maastricht	57	46.7	23	62.6

[a] Kaplan–Meier survival estimate.
[b] Radiotherapy.
[c] Chemotherapy.
[d] 22 fractions daily, last 12 fractions twice daily.
[e] Of (chemo)radiotherapy.

thesis genes (*Drosha*: rs639174, rs3805500, rs10520985, rs17410035; *XPO5*: rs2227301, rs699937) and two polymorphisms in miRNA target genes (*KRAS*: rs61764370; *SMC1B*: rs3747238) were considered. Genomic DNA was obtained from blood using the Puregene genomic DNA purification kit (Gentra systems). Genotyping was performed using restriction fragment length polymorphism (RFLP) analyses, high resolution melting (HRM) curve analyses and/or direct sequencing. Primer sequences and restriction enzymes are available in the online Tables S1 and S2. For reproducibility control, 15% of all samples were duplicated. The concordance rate between duplicate samples was 100%. The maximal number of missing values per polymorphism was three.

2.4. Statistical methods

Five-year survival was designated as a clinically relevant endpoint. Overall survival time was calculated from the date of primary treatment to the date of death from any cause; survivors were censored at their last follow-up. Disease specific survival time was calculated from the date of primary treatment to the date of death from disease; patients who died of other/unknown cause were censored at time of death, survivors were censored at their last follow-up. Patients that were lost from follow-up were censored. Survival plots and 5-year survival rates were obtained by Kaplan–Meier analysis, with comparisons between groups made by the log-rank test. The Cox proportional hazard model was used to estimate hazard ratio's (HR) characterizing the independent prognostic significance of single and multiple variables. To account for missing values, multiple imputation was used. Therefore, 100 datasets were imputed which allow for efficient computation of *p*-values. The best multivariate model was chosen by stepwise regression (both forward and backward) that minimizes the AIC (Akiake Information Criterion) score. Traditional methods that add significant terms based on *F*-statistics fail to account for multiple testing and hence do not capture important markers. Standard errors and *p*-values of the variables are derived from a combination of standard errors from sample variation and variation due to multiple imputation. All analyses were performed in R by applying the R package mi for multiple imputation [20] and the survival package for Cox modelling [21]. Differences in distributions of selected characteristics between HPV-positive and HPV-negative patients were evaluated using χ^2 or Mann–Whitney test in the SPPS software packet.

3. Results

3.1. Patient characteristics

The 122 patients included in this study (Table 1) were predominantly male (81.1%), ever smokers with more than 10 pack-years (78.9%) and represented primarily stage III/IV tumours (88.5%). The overall median follow-up time was 46.7 months (range 2.4–119.7 months), during which period 59 patients died from any cause, 32 patients relapsed (local, regional or distant) and 27 patients died from the disease. The overall median follow-up time was 60.0 months for surviving patients (range 18.1–112.9 months) and 19.3 months for deceased patients (range 2.4–119.7 months). Five years overall survival and disease specific survival was 55% and 76%, respectively.

3.2. Characteristics of the studied genetic polymorphisms

Ten genetic polymorphisms were determined for all patients. The minor allele frequencies of the *pre-mir-146a* rs2910164 and the *pre-mir-196a2* rs11614913 polymorphisms were 29.2% and 40.0%, respectively. The minor allele frequencies of the *Drosha*

rs639174, rs3805500, rs10520985 and rs17410035 polymorphisms were 20.4%, 32.5%, 47.5% and 30.0%, respectively. For the *XPO5* rs2227301 and rs699937 polymorphisms, the *KRAS* rs61764370 and the *SMC1B* rs3747238 polymorphism, the minor allele frequencies were 26.5%, 33.3%, 9.6% and 40.4%, respectively. All genotype distributions were in Hardy–Weinberg equilibrium ($p > 0.156$). The genotype frequencies of the studied genetic polymorphisms in relation to outcome are presented in Table 2. In univariate analysis (log-rank and logistic regression) none of the considered genetic polymorphisms were significantly associated with overall survival (data not shown). As we aimed at considering all studied variables from the start of the analysis and at accounting for missing values and multiple testing, the methodology as described in Section 2.4 was used for variable selection and estimation of hazard ratios.

3.3. Multivariate analysis for survival

Multivariate Cox analysis including all clinical, treatment and genetic parameters was performed for 5-years overall survival (Table 3). A similar analysis for disease specific survival was unfeasible due to the low number of disease-related deaths. HPV negativity (HR = 3.89, $p = 0.005$), advanced T-stage (HR = 1.81, $p = 0.050$), advanced N-stage (HR = 5.86, $p = 0.001$) and >10 pack-years of smoking (HR = 3.45, $p = 0.012$) were significantly associated with reduced overall survival. Of all treatment factors included in the analysis, IMRT (HR = 2.27, $p = 0.028$) and longer treatment times (HR = 1.78, $p = 0.050$) were associated with an increased hazard of death. The wild-type genotype of the *KRAS-LCS6* rs61764370 polymorphism (TT) was significantly associated with reduced overall survival (HR = 2.47, $p = 0.031$). None of the other studied genetic polymorphisms were significantly associated with survival. The Kaplan–Meier plot in Fig. 1 demonstrates the added value, next to HPV status, of the *KRAS-LCS6* polymorphism as prognostic marker in especially HPV$^-$ OPC patients. For patients with HPV$^-$ tumours, overall survival at 5 years was 60% for TG genotypes and 42% for TT genotypes ($p = 0.050$). A similar survival plot by HPV status and pack-years of smoking shows that HPV$^-$ patients with ≤10 pack-years had a mean 5-year overall survival rate comparable to HPV$^+$ patients, whereas a firm decrease in overall survival was seen for HPV$^-$ patients with >10 pack-years (Fig. 1).

3.4. HPV prevalence and association with selected variables

Tumours were classified as HPV-positive if they overexpressed p16 and contained DNA from high-risk HPV types (by PCR-EIA or ISH). The overall HPV prevalence rate was 27.9% (34/122) and the majority of infections (30/34) occurred in tonsil or base of tongue subsites (prevalence rates of 36.4% and 40.0%, respectively). Among cases that tested positive for p16 IHC (41/122), 7 were negative for ISH (4/7) or PCR-EIA (3/7). This overestimation by p16 IHC of 17% is in accordance with literature [22]. None of the tumours were p16-negative/ISH-positive. Examples of p16 IHC/HPV ISH positive and negative cases are given in Fig. 2.

The distribution of selected variables by tumour HPV status is presented in Table 4. There was no difference in age and gender between HPV$^+$ and HPV$^-$ OPSCC patients. Compared to HPV$^-$ patients, HPV$^+$ patients had less tobacco (>10 pack-years: 51.9% *vs.* 87.8%, $p < 0.001$) and alcohol exposure (≥10 drinks/week: 28.1% *vs.* 75.6%, $p < 0.001$). More HPV$^+$ patients had lower T-stages ($p = 0.079$) and HPV$^+$ tumours occurred more frequently in the tonsils ($p = 0.058$). There were no differences in treatment regimens between HPV$^+$ and HPV$^-$ patients. HPV$^+$ patients had a better 5-years overall survival (78% *vs.* 46%, $p = 0.001$) and a better 5-years disease specific survival (90% *vs.* 70%, $p = 0.016$) compared to HPV$^-$ patients. The HPV$^+$ ratio was significantly higher in Maastricht compared to Gent (36.8% *vs.* 20.0%, $p = 0.038$) and increased over time (38.5% before *vs.* 15.8% since 2007, $p = 0.005$).

Table 2
Genotype frequencies of the studied genetic polymorphisms in relation to outcome.

	n	%	Overall survival	
	122		Deaths	% surviving 5 years[a]
pre-mir-146a rs2910164				
GG	57	47.5	29	49.5
GC	56	46.7	26	60.4
CC	7	5.8	3	57.1
Missing	2			
MAF[b]		29.2		
pre-mir-196a2 rs11614913				
CC	45	37.5	23	57.9
CT	54	45.0	26	48.8
TT	21	17.5	9	63.8
Missing	2			
MAF[b]		40.0		
Drosha rs639174				
GG	74	61.7	36	53.7
GA	43	35.8	20	57.1
AA	3	2.5	2	66.7
Missing	2			
MAF[b]		20.4		
Drosha rs3805500				
TT	54	45.0	28	54.9
TC	54	45.0	24	57.5
CC	12	10.0	6	43.7
Missing	2			
MAF[b]		32.5		
Drosha rs10520985				
CC	35	29.2	15	58.9
CT	56	46.7	28	51.7
TT	29	24.2	15	57.0
Missing	2			
MAF[b]		47.5		
Drosha rs17410035				
CC	58	48.3	28	56.0
CA	52	43.3	26	53.8
AA	10	8.3	4	60.0
Missing	2			
MAF[b]		30.0		
XPO5 rs2227301				
GG	63	52.9	36	44.1
GA	49	41.2	19	63.5
AA	7	5.9	3	68.6
Missing	3			
MAF[b]		26.5		
XPO5 rs699937				
CC	52	43.3	30	45.6
CT	56	46.7	22	59.4
TT	12	10.0	6	63.5
Missing	2			
MAF[b]		33.3		
KRAS rs61764370				
TT	97	80.8	46	52.9
TG	23	19.2	12	62.9
GG	0	0	0	–
Missing	2			
MAF[b]		9.6		
SMC1B rs3747238				
AA	40	33.3	18	54.5
AG	63	52.5	32	53.6
GG	17	14.2	8	52.9
Missing	2			
MAF[b]		40.4		

[a] Kaplan–Meier survival estimate.
[b] Minor allele frequency.

Table 3
Multivariate analysis for risk of death from any cause.

	Subgroups	Overall survival		
		HR	95% CI	*p*-Value
Pack-years	>10PY *vs.* ≤10 PY	3.45	1.32–9.09	0.012
T-stage	3/4 *vs.* 1/2	1.81	1.00–3.28	0.050
N-stage	2b/2c/3 *vs.* 0/1/2a	5.86	2.61–13.14	0.001
HPV status	Negative *vs.* positive	3.89	1.51–10.00	0.005
Radiotherapy type technique	IMRT *vs.* 3DCRT	2.27	1.09–4.72	0.028
Treatment time[a]		1.78	1.00–3.17	0.050
KRAS-LCS6 rs61764370	TT *vs.* TG	2.47	1.09–5.62	0.031

[a] Of (chemo)radiotherapy, continuous variable.

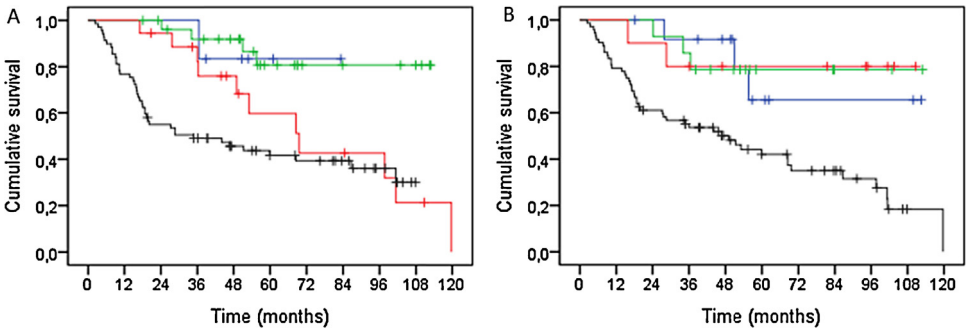

Fig. 1. Kaplan–Meier plots. (A) Overall survival by HPV status and *KRAS-LCS* genotype. Blue line: HPV$^+$ and TG genotype (n = 6), green line: HPV$^+$ and TT genotype (n = 27), red line: HPV$^-$ and TG genotype (n = 18), black line: HPV$^-$ and TT genotype (n = 69). HPV$^-$ and TG genotype (red line) compared to HPV$^-$ and TT genotype (black line) at 5 years: p = 0.050. (B) Overall survival by HPV status and pack-years of smoking. Blue line: HPV$^+$ and ≤10 pack-years (n = 13), green line: HPV$^+$ and >10 pack-years (n = 14), red line: HPV$^-$ and ≤10 pack-years (n = 10), black line: HPV$^-$ and >10 pack-years (n = 72). HPV$^-$ and ≤10 pack-years (red line) compared to HPV$^-$ and >10 pack-years (black line) at 5 years: p = 0.056. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of the article.)

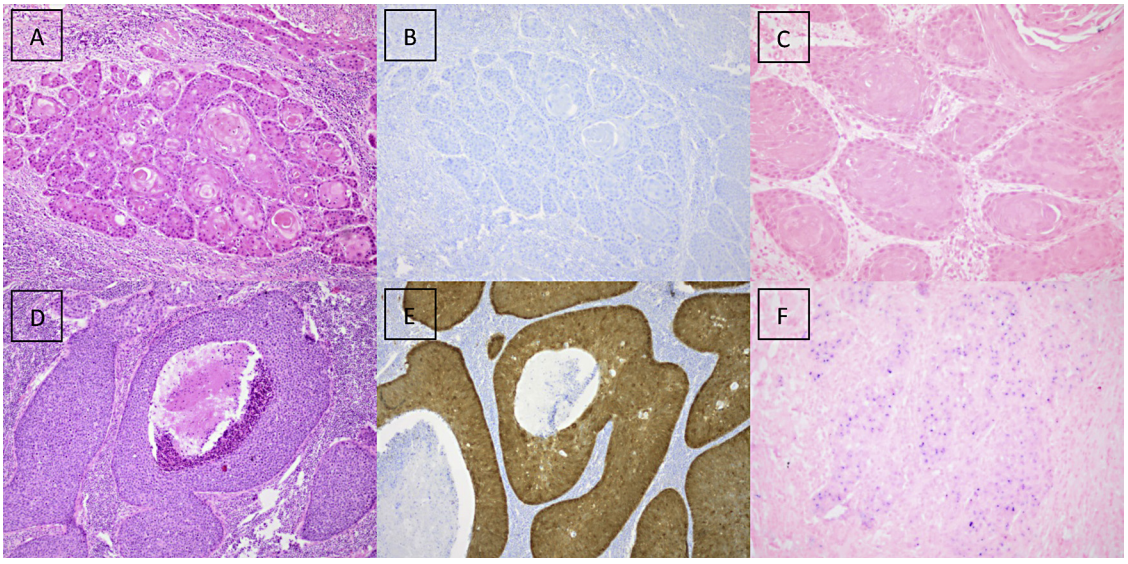

Fig. 2. Cases of OPSCC evaluated by HE staining (A and D), p16 IHC (B and E) and high-risk HPV ISH (C and F). (A–C) An example of a p16-negative (B) and high-risk HPV ISH-negative (C) case. (D–F) An example of a p16-positive (E) and high-risk HPV ISH-positive (F) case. Magnifications: 100× (A, B, D, E), 200× (C and F).

The cut-off was based on the median start date of radiotherapy. HPV prevalence over time for the two geographical locations is further illustrated in Fig. 3.

4. Discussion

The survival of patients with advanced OPSCC may differ significantly between individuals. The ability to estimate survival probability before any type of treatment would be very valuable for decision making, as well as for developing clinical trials. Prior studies have demonstrated that positive HPV status confers an enormous prognostic benefit to patients. As additional biomarkers may help in refining patient stratification, we investigated the prognostic value of miRNA, miRNA-binding site and miRNA processing gene polymorphisms in OPC patients.

In our dataset, nodal stage, HPV status and smoking quantity were found to have the strongest impact on survival. Next to these known prognostic factors, the considered *KRAS-LCS6* polymorphism was also significantly associated with prognosis of OPSCC. *KRAS* is a proto-oncogene that is activated by somatic mutation in many human cancers. *KRAS* mutation is more prevalent in colorectal (31%) and non-small cell lung (19%) cancer compared

Table 4
Distribution of selected variables by tumour HPV status.

	HPV positive pts ($n=34$)		HPV negative pts ($n=88$)		p-Value
	Number	%	Number	%	
Gender					
Men	29	85.3	70	79.5	
Woman	5	14.7	18	20.5	0.467
Age					
Median	61.8	–	60.2	–	
Range	37.9–77.9	–	37.2–77.3	–	0.756
Pack-years					
≤10	13	48.1	10	12.2	
>10	14	51.9	72	87.8	<0.001
Drinks/week					
<10	23	71.9	21	24.4	
≥10	9	28.1	65	75.6	<0.001
Subsite tumour					
Tonsil	20	58.8	35	39.8	
Other	14	41.2	53	60.2	0.058
T-stage					
1–2	23	67.6	44	50.0	
3–4	11	32.4	44	50.0	0.079
N-stage					
0–1–2a	12	35.3	33	38.4	
2b–2c–3	22	64.7	53	61.6	0.754
Treatment					
RT[a]	12	35.3	36	40.9	
RT[b] + ChT[b]	14	41.2	35	39.8	
Surgery + RT[a]	4	11.8	13	14.8	
Surgery + RT[a] + ChT[b]	4	11.8	4	4.5	0.509
Centre					
Gent	13	38.2	52	59.1	
Maastricht	21	61.8	36	40.9	0.038
Time span					
2003–2006	9	26.5	48	54.5	
2007–2012	25	73.5	40	45.5	0.005

[a] Radiotherapy.
[b] Chemotherapy.

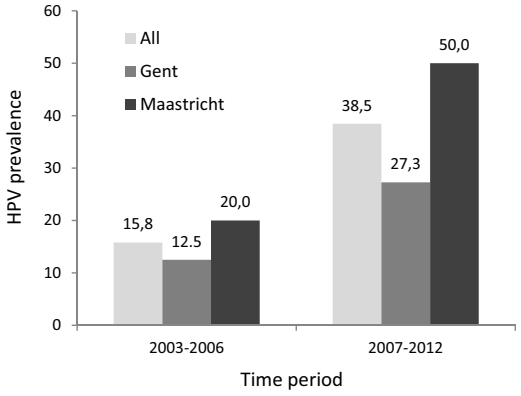

Fig. 3. HPV prevalence over time for the two geographical locations.

to oral and tonsil cancer (4%) [23,24]. In addition to gene mutation, KRAS activity can be altered by the let-7 family of miRNAs. It has been shown that let-7 induces RAS downregulation after binding to specific sites in the 3′-UTR *KRAS* mRNA [25,26]. The rs61764370 polymorphism in *KRAS-LCS6* affects let-7-mediated regulation of KRAS expression. In lung cancer cell lines, the variant G-allele was observed to lead to higher KRAS levels and lower let-7 levels as compared to the wild type [27]. A reduction in let-7 miRNA expression levels was also observed in a colorectal cancer cell line harbouring the variant G-allele but no change in KRAS protein expression was observed [28]. G-allele carriers have been shown to have an increased lung cancer risk in moderate smokers [27], an increased ovarian cancer risk [29] and an increased triple-negative breast cancer risk [30]. Furthermore, the variant G-allele has been associated with reduced survival in oral cancer but not in pharyngeal, lung and ovarian cancer [15,31,32]. For colorectal cancer, both Smits et al. [33] and Ryan et al. [34] reported a better survival for patients with the variant G-allele, albeit Smits et al. only for early-stage tumours and Ryan et al. only for late-stage tumours. For the latter studies, differences in *KRAS* mutation and therapy could have modulated the impact of the polymorphism. In the present study, we also found a significant survival advantage for G-allele carriers in our oropharyngeal study population. As mentioned above, Christensen et al. did not observe an altered survival pattern for pharyngeal cancer patients – which were not exclusively oropharyngeal – in relation to the *KRAS* polymorphism, but did find a worse prognosis for oral cancer cases with the *KRAS* polymorphism [15]. As normal oral and pharyngeal epithelium are different cell types with specific transcriptomes, alterations in let-7 family miRNA expression will impact expression of miRNA target transcripts in a cell type specific way. Hence, differential dysregulation of miRNA targets could explain the differential findings between both studies.

One of the remarkable findings was that patients treated with non-IMRT techniques had a survival advantage compared to patients treated with IMRT. This can be explained by the interaction of treatment type with T- and N-stage; more patients with higher stadia were treated with IMRT. Furthermore, patients with shorter treatment times had significantly better survival rates. Of all factors possibly influencing treatment time, only fractionation scheme was significantly associated with it (mean TT_{daily} = 45.0 days, mean TT_{hybrid} = 36.8 days, $p < 0.001$). Patients receiving a hybrid fractionation scheme indeed had a better 5-years overall survival (65% *vs.* 51%, $p = 0.184$) and a better 5-years disease specific survival (92% *vs.* 71%, $p = 0.026$) compared to patients receiving daily fractionations. Despite the fact that in current dataset more patients with small tumours were treated with a hybrid fractionation scheme, it is well-known that shortening of treatment time reduces accelerated proliferation in tumours and improves outcome [35].

As HPV prevalence rates differ between different geographical regions and time periods, we wanted to map the prevalence of HPV-associated OPC in a Belgian/Dutch cohort. The prevalence rate in this study was 28% for all patients, 20% for patients in the North of Belgium and 37% for patients in the South of the Netherlands. The large difference between sites was not caused by selection bias. Oropharyngeal cancer caused by HPV increased from 16% in 2003–2006 to 39% in 2007–2012. These rates are comparable to a Dutch series (19%, 2000–2006, [36]) but significantly lower than UK (55%, 2001–2006, [37]) and US (59%, 2002–2005, [7]) series. Considering the rapid increase in prevalence in our dataset, it is expected that prevalence rates resembling those of other regions will be reached between 10 and 20 years.

In conclusion, HPV was detected in 28% of OPSSC in this study. OPC patients with the *KRAS-LCS6* variant allele showed to have a survival advantage suggesting that, if externally validated, this variant may be used as an additional prognostic marker for OPSCC.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Authorship contribution

Kim De Ruyck: contributions to the conception, design, acquisition, analysis and interpretation of data; drafting of the manuscript; final approval of the manuscript.

Fréderic Duprez: contributions to the acquisition and interpretation of data; critical manuscript revising, final approval of the manuscript.

Liesbeth Ferdinande: contributions to the design and acquisition; critical manuscript revising; final approval of the manuscript.

Chamberlain Mbah: substantial contributions to the analysis; critical manuscript revising; final approval of the manuscript.

Emmanuel Rios Velazquez: contributions to the acquisition; critical manuscript revising; final approval of the manuscript.

Frank Hoebers: contributions to the acquisition and interpretation of data; critical manuscript revising; final approval of the manuscript.

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Philippe Deron: contributions to the acquisition; critical manuscript revising; final approval of the manuscript.

Katrien Bonte: contributions to the acquisition; critical manuscript revising, final approval of the manuscript.

Ernst-Jan Speel: contributions to the acquisition and interpretation of data; critical manuscript revising; final approval of the manuscript.

Louis Libbrecht: contributions to the interpretation of data; critical manuscript revising; final approval of the manuscript.

Wilfried De Neve: contributions to the interpretation of data; critical manuscript revising; final approval of the manuscript.

Philippe Lambin: contributions to the conception and interpretation of data; critical manuscript revising; final approval of the manuscript.

Hubert Thierens: contributions to the conception, design and interpretation of data; critical manuscript revising; final approval of the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canep.2014.07.008

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