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HPV-negative squamous cell carcinoma of the anal canal is unresponsive to standard treatment and frequently carries disruptive mutations in *TP53*

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Background: Human papillomavirus (HPV), p16 expression, and *TP53* mutations are known prognostic factors in head and neck squamous cell carcinoma, but their role in squamous cell carcinoma of the anal canal (SCCAC) is less well established. The objective of this study was to determine the prognostic significance of tumour HPV status, p16 and p53 expression, and mutations in *TP53* in patients with SCCAC receiving (chemo)radiotherapy.

Methods: Human papillomavirus DNA was determined using an INNO-LiPA-based assay in tumour tissue of 107 patients with locally advanced SCCAC. Patients were treated with radiotherapy, with or without concurrent chemotherapy consisting of a fluoropyrimidine and mitomycin C. Expression of p16 and p53 was determined using immunohistochemistry. Exons 2–11 of *TP53* in tumour tissue were sequenced.

Results: DNA of high-risk HPV types was detected in 93 out of 107 tumours (87%), all of which overexpressed p16 (HPV+/p16+). Of 14 HPV-negative (HPV−) tumours (13%), 10 (9%) were p16-negative (HPV−/p16−) and 4 (4%) overexpressed p16 (HPV−/p16+). Patients with HPV−/p16− disease had inferior 3-year locoregional control (LRC) (15%) compared with patients with HPV+/p16+ tumours (82%, $P<0.001$) and HPV−/p16+ tumours (75%, $P=0.078$). Similarly, 3-year overall survival (OS) was 35% (HPV−/p16−) *vs* 87% (HPV+/p16+, $P<0.001$) and 75% (HPV−/p16+, $P=0.219$). Disruptive mutations in *TP53* were found in 80% of HPV−/p16− tumours *vs* 6% of HPV+/p16+ tumours ($P<0.001$). In multivariate analysis, HPV−/p16− status was an independent predictor of inferior LRC and OS.

Conclusions: HPV− tumours are frequently *TP53* mutated. HPV−/p16− status is a strong predictor for reduced LRC and OS, and alternative treatment strategies for patients with HPV−/p16− disease need to be explored.

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Squamous cell carcinoma of the anal canal (SCCAC) is a relatively rare malignancy with 1–2 per 100 000 new cases in Europe and the United States each year, but its incidence is rising (Netherlands Cancer Registry; Nelson *et al*, 2013). Most patients present with locally advanced, non-metastasised disease, for which the established treatment is concurrent chemoradiotherapy, consisting of radiotherapy, combined with 5-fluorouracil (5-FU) and mitomycin C (MMC) (James *et al*, 2013). With standard treatment, complete and durable remission can be achieved in the majority of patients, with clinical complete response rates as high as 90%, locoregional control (LRC) at 3 years of 70–80% and 3-year overall survival (OS) of 80–85% (James *et al*, 2013). Ten to twenty per cent of the patients, however, do not respond to therapy or relapse early after treatment. Although several prognostic factors for unfavourable outcome have been identified – including male sex and high T- and N-classification (Ajani *et al*, 2010) – these factors do not fully explain differences in outcome. Squamous cell carcinoma of the anal canal is known to be strongly linked with the human papillomavirus (HPV), a small double-stranded DNA virus commonly known for its role in the development of cervical cancer, other gynaecological cancers, and head and neck squamous cell carcinoma (HNSCC). The prevalence of high-risk HPV (HR-HPV) types in SCCAC – that is, those types associated with carcinogenesis – ranges from 70 to almost 100%, depending on the population studied and the sensitivity of the method used for HPV detection (Daling *et al*, 2004; Hoots *et al*, 2009; Valmary-Degano *et al*, 2013). Human papillomavirus can induce carcinogenesis via expression of the oncoproteins E6 and E7, which act by inactivating the tumour suppressor proteins p53 and retinoblastoma protein (pRb), respectively. Persisting inactivation of p53 and pRb leads to genomic instability and, upon persisting infection, to carcinogenesis. As pRb is a negative regulator of the cyclin-dependent kinase inhibitor p16, inactivation of pRb by HPV leads to marked upregulation of p16. For this reason, p16 overexpression, measured by immunohistochemistry, is often used as a surrogate marker for tumour HPV infection. A proportion of SCCAC tumours are found to be HPV-negative (HPV−), that is, they do not carry HPV, or carry only low-risk HPV (LR-HPV) types – that is, types that are generally not associated with carcinogenesis. It has been demonstrated in patients with HNSCC that HPV− tumours have substantially worse outcome compared with patients with HPV+ tumours. Overall survival at 3 years was 82% for patients with HPV+ disease, compared with 57% for patients with HPV− disease ($P<0.001$) (Ang *et al*, 2010). Also in cervical cancer, HPV− status is an unfavourable prognostic factor (Harima *et al*, 2002). The considerable differences in outcome of patients with HPV+ *vs* HPV− tumours have been attributed to differences in tumour biology. Indeed, in HNSCC there are marked differences between HPV+ and HPV− tumours at the level of mutational patterns, loss-of-heterozygosity, and chromosomal alterations (Braakhuis *et al*, 2004; Smeets *et al*, 2006; Stransky *et al*, 2011; Lechner *et al*, 2013). These differences might affect prognosis and effectiveness of treatment. A particularly striking difference between HPV+ and HPV− HNSCC tumours are mutations in *TP53* (the gene encoding p53). Whereas *TP53* mutations are found only sporadically in HPV+ HNSCC tumours (0–10%), they occur very frequently in HPV− tumours, with disruptive mutations present in 80–100% of cases (Westra *et al*, 2008; Agrawal *et al*, 2011; Stransky *et al*, 2011; Lechner *et al*, 2013). *TP53* mutations are of interest in particular, because they have been linked to prognosis in different types of cancer (Tandon *et al*, 2010; Stengel *et al*, 2014) and, in addition, to resistance to radiation therapy (Skinner *et al*, 2012; Kimple *et al*, 2013), which is an important treatment modality for SCCAC and most other HPV-associated cancers. To investigate the prognostic significance of HPV, p16, and alterations in p53 function in SCCAC, we determined associations with outcome of tumour HPV status, p16 expression, p53 expression, and mutations in *TP53* in a cohort of 107 anal carcinoma patients treated with chemoradiotherapy or radiotherapy alone.

MATERIALS AND METHODS

Patients. All consecutive patients, ⩾18 years of age, with histologically confirmed locally advanced SCCAC, treated at our institute between August 2003 and August 2011 with chemoradiotherapy or radiotherapy alone were included. Patients with T2–4 (T⩾4 cm), N0–1, M0 or T1–4, N2–3, M0 tumours were treated with concurrent chemoradiotherapy with a fluoropyrimidine (5-FU or capecitabine), MMC (10 mg m^{-2} on day 1), and three-field conformal RT or IMRT. Patients with T1–2 (T⩾1 and <4 cm), N0–1, M0 disease were treated with radiotherapy alone. Detailed treatment characteristics and inclusion criteria were reported previously (Meulendijks *et al*, 2014). In addition, patients with no paraffin-embedded tumour biopsy tissue available were excluded. Data collection was approved by the institutional ethics committee.

HPV DNA isolation, amplification, and genotyping. Formalin-fixed paraffin-embedded (FFPE) tissue samples were sectioned to avoid contamination with HPV DNA from tissue to tissue. DNA was isolated from three 8-μm sections using the cobas DNA Sample Preparation Kit (Roche Diagnostics, Indianapolis, IN, USA). Subsequently, HPV DNA was amplified using the SPF10 PCR primer set from the INNO-LiPA HPV Genotyping Extra Kit (Fujirebio Europe, Gent, Belgium). During each run, a negative control (water) and a positive control (HPV18+ sample) was analysed. SPF10 amplimers from HPV DNA-positive samples were subsequently analysed using an HPV Line Probe assay (LiPA) (Kleter *et al*, 1999). Here also, positive and negative controls were included in each analytical run. Human papillomavirus 6, 11, 40, 43, 44, 54, 71, and 74 were considered LR-HPV types, whereas HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 70, 73, and 82 were considered HR-HPV types. To minimise the risk of false-negative results, all samples that were HPV− were reanalysed with INNO-LiPA, and subsequently analysed with a second test, RealTime High Risk HPV (Abbott Diagnostics, Lake Forest, IL, USA).

Immunohistochemistry of p16 and p53. Immunohistochemistry (IHC) was performed on a BenchMark ULTRA autostainer (Ventana Medical Systems, Tucson, AZ, USA). Briefly, 4-µm paraffin sections were cut, heated for 28 min at 75 °C, and deparaffinised in the instrument using EZ prep solution (Ventana Medical Systems). Heat-induced antigen retrieval was carried out using Cell Conditioning 1 (Ventana Medical Systems) for 36 min at 95 °C. To detect p16, sections were incubated for 32 min with antibody clone JC8 (ILM8763-C1; ImmunoLogic, Duiven, the Netherlands). For p53, antibody clone DO-7 (M7001; Dako, Glostrup, Denmark) was used (incubation 32 min). After incubation with the primary antibodies, the Amplification Kit (Ventana Medical Systems) was applied. Specific reactions were detected using UltraView Universal DAB Detection Kit (Ventana Medical Systems), and slides were counterstained with haematoxylin. Protein expression scoring was performed by two investigators (MFV and DM) who were not aware of patient identity. In case of discrepant scoring, consensus was reached between the investigators. Staining of p16 was scored as positive (p16+) when ⩾70% of the tumour cells showed strong nuclear staining, as opposed to negative in case <70% of cells showed strong nuclear staining (p16−) (Ang *et al*, 2010). Expression of p53 was scored as non-aberrant when 1–70% of cells showed strong nuclear staining, or as aberrant when either >70% or 0% of the tumour cells showed strong nuclear staining (Yemelyanova *et al*, 2011).

***TP53* mutational analysis.** *TP53* mutational analysis was performed in all patients with HPV− tumours ($n=14$), and 18 randomly selected patients with HPV+ disease. Exons 2–11 of *TP53* were amplified using PCR on genomic DNA derived from FFPE tumour tissue (primer sequences available upon request). Purified PCR reaction products were then sequenced using BigDye Terminator v.1.1 (Applied Biosystems, Foster City, CA, USA). The sequence fragments were analysed using an automated sequencer (ABI3730; Applied Biosystems) and data were analysed using Mutation Surveyor (Softgenetics LLC, State College, PA, USA). The *TP53* database of the International Agency for Research on Cancer (IARC, Lyon, France; http://p53.iarc.fr) was used to determine the functional consequences of *TP53* mutations. Mutations without functional consequences were considered non-disruptive; mutations resulting in a completely or partially dysfunctional protein were grouped as disruptive mutations.

End points and statistical analysis. Patient demographics and disease characteristics of groups according to HPV/p16 status were described by means of descriptive statistics and compared using Student's *t*-test (age), Mann–Whitney *U*-test (T-classification, N-classification, UICC stage), or Fisher's exact test (gender, SCC antigen, HIV status, smoking status). The primary end points of the study were LRC and OS, as a function of tumour HPV status, p16 expression, p53 expression, and *TP53* mutational status. Thus, patients were grouped based on the presence or absence of HR-HPV in tumour (HPV+ *vs* HPV−), p16 expression (p16+ *vs* p16−), p53 expression (non-aberrant *vs* aberrant expression), and the presence of disruptive mutations in *TP53* (disruptive mutation present *vs* absent). Locoregional control and OS were defined as the time between the first day of treatment and the day on which clinical signs of progression (at the primary site or regional, inguinal, or pelvic lymph nodes) or death of any cause occurred, respectively. Locoregional control and OS were compared between groups using log-rank tests. For multivariate analysis of factors related to outcome, a Cox regression model was used in which covariates that were significant upon univariate analysis were included. *TP53* mutations were not included in the multivariate analysis as only a limited number of tumours was analysed. The influence of HPV/p16 status and p53 expression on outcome was analysed separately in the entire cohort and in the subgroup of patients with early-stage tumours treated with radiotherapy alone. All statistical tests were two-sided, with the threshold for significance set at $P<0.05$. Statistical analyses were performed using SPSS version 17.0 (SPSS, Chicago, IL, USA).

RESULTS

Patients. Of 138 patients treated between August 2003 and August 2011, 107 met the in- and exclusion criteria. Of all tumours, 93 were found to be HPV+/p16+ (87%), 4 were HPV−/p16+ (4%), and 10 were HPV−/p16− (9%). Patient characteristics according to HPV/p16 status are shown in Table 1. Patients with HPV− tumours were more often males compared with patients with HPV+ tumours (86% *vs* 44%, $P=0.003$). The individual groups of HPV−/p16+ and HPV−/p16− patients were also more often males than patients with HPV+/p16+ tumours ($P=0.032$ and $P=0.019$, respectively). T-classification, N-classification, and UICC stage did not differ significantly between groups, although HPV− tumours tended to have higher T-classification ($P=0.074$).

HPV genotyping. All 107 tissues could be genotyped for HPV DNA. The detected HPV types are summarised in Table 2. As expected, HPV16 was the most prevalent HR-HPV type, accounting for 81 out of 93 (87%) of all HPV+ tumours. Based on the results of the INNO-LiPA test, initially six discordant cases (HPV−/p16+) were identified. These were reanalysed with INNO-LiPA, and subsequently with the secondary HPV test. Four out of six tumours were confirmed to be HPV−, whereas two cases showed to be positive on reanalysis with the second assay (one HPV18+ case and one case HR-HPV+ not otherwise specified).

Immunohistochemistry of p16 and p53. All HR-HPV+ tumours were also p16+, which included 93 out of 107 patients (85%). Of the remaining 14 HPV-negative tumours, 10 out of 14 were HPV−/p16−, and 4 out of 14 were HPV−/p16+. The four tumours carrying the LR-HPV type 6, shown in Table 2, were all p16−. Overall, 14 out of 107 tumours (13%) showed aberrant p53 expression patterns, of which two showed 0% staining (both were HPV−/p16−). Aberrant p53 expression was more frequent in HPV−/p16− tumours (5 out of 10, 50%) than in HPV+/p16+ tumours (9 out of 93, 10%, $P=0.004$; Figure 1A). Among the HPV−/p16+ tumours, 0 (0%) had aberrant p53 expression. Figures of representative immunohistochemical stainings for p16 and p53 are available in the Supplementary Material (Supplementary Figure S1).

***TP53* mutational analyses.** Sequences of exons 2–11 of *TP53* could be determined in 31 of (all) 14 HPV− and 18 selected HPV+ samples. DNA quality of one sample (of a HPV−/p16+ patient) did not permit reliable interpretation of results. The distribution of disruptive *TP53* mutations according to HPV/p16 status is depicted in Figure 1B, showing that mutations were far more prevalent in HPV− tumours than in HPV+ tumours. Disruptive mutations were found in 1 out of 18 (6%) HPV+/p16+ tumours, 1 out of 3 (33%) HPV−/p16+, and 8 out of 10 (80%) HPV−/p16− tumours. In addition, in the HPV+/p16+ group one mutation that is classified as non-disruptive was detected. The detected variants are summarised in Table 3. Of six tumours that had aberrant expression on IHC, 5 out of 6 were *TP53* mutation positive (83%). Of 26 tumours that were non-aberrant on IHC, 20 (77%) did not carry a *TP53* mutation. Of 10 tumours that carried a *TP53* mutation, five were aberrant on IHC (50%).

Outcome in relation to HPV status and p16 expression. Locoregional control and OS were strongly influenced by HPV/p16 status. Figure 2 shows the outcome of patients according to HPV/p16 status. Patients with HPV−/p16− tumours had significantly inferior 3-year LRC (15%) compared with HPV+/p16+ tumours (82%, $P<0.001$) and HPV−/p16+ tumours (75%, $P=0.078$). Overall survival at 3 years was 35% for patients with HPV−/p16− tumours compared with 87% for HPV+/p16+ tumours ($P<0.001$ compared with HPV−/p16−) and 75% for HPV−/p16+ ($P=0.219$ compared with HPV−/p16−). Among the patients who died during follow-up, in the HPV−/p16− group 5 out of 6 patients (83%) died owing to anal cancer, and in the HPV+/p16+ group, 7 out of 11 patients (64%). The only deceased patient in the HPV−/p16+ group died of anal cancer. We did not identify distinct patterns of local or distant recurrence for HPV+ *vs* HPV− tumours. Distant recurrence occurred in seven patients (8%) in the HPV+/p16+ group (distant lymph nodes, four patients; liver, two patients; lung, one patient), and in 1 out of 16 patients (6%) in the HPV− group (skeletal). In the subgroup of patients with early-stage tumours who received radiotherapy alone, LRC and OS were also strongly influenced by HPV/p16 status (Supplementary Figure S2). Locoregional control at 3 years was 0% for patients with HPV−/p16− tumours compared with 85% for HPV+/p16+ tumours ($P<0.001$). Similarly, OS was 33% *vs* 100% ($P=0.002$). There were no HPV−/p16+ patients treated with radiotherapy alone.

Outcome in relation to p53 expression and *TP53* alterations. Both aberrant p53 expression and *TP53* mutations were associated

Table 1. Patient and disease characteristics by HPV and p16 status

Characteristic	Overall ($n=107$)	HPV+/p16+ ($n=93$)	HPV−/p16+ ($n=4$)	HPV−/p16− ($n=10$)	*P*-value (HPV+ *vs* HPV−)
Age, median (range)[a]	60 (34–86)	60 (34–86)	58 (43–70)	61 (46–74)	0.894
Sex					
Male	50 (47%)	38 (41%)	4 (100%)	8 (80%)	0.003
Female	57 (53%)	55 (59%)	0 (0%)	2 (20%)	
T-classification					
T1	4 (4%)	4 (4%)	0 (0%)	0 (0%)	0.074
T2	53 (50%)	49 (53%)	0 (0%)	4 (40%)	
T3	32 (30%)	25 (27%)	4 (100%)	3 (30%)	
T4	18 (17%)	15 (16%)	0 (0%)	3 (30%)	
N-classification					
N0	49 (46%)	40 (43%)	3 (75%)	6 (60%)	0.383
N1	30 (28%)	30 (32%)	0 (0%)	0 (0%)	
N2	18 (17%)	13 (14%)	1 (25%)	4 (40%)	
N3	9 (8%)	9 (10%)	0 (0%)	0 (0%)	
Nx	1 (1%)	1 (1%)	0 (0%)	0 (0%)	
UICC stage					
Stage 0	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0.429
Stage I	5 (5%)	5 (5%)	0 (0%)	0 (0%)	
Stage II	36 (34%)	29 (31%)	3 (75%)	4 (40%)	
Stage III	65 (61%)	58 (62%)	1 (25%)	6 (60%)	
Stage IV	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
Not known	1 (1%)	1 (1%)	0 (0%)	0 (0%)	
HIV status					
Negative	34 (31%)	27 (30%)	3 (75%)	2 (20%)	0.573
Positive	10 (9%)	10 (11%)	0 (0%)	0 (0%)	
Not known	63 (59%)	54 (59%)	1 (25%)	8 (80%)	
Smoking status					
Smoker	31 (29%)	26 (28%)	1 (25%)	4 (40%)	1.000[b]
Non-smoker	9 (8%)	8 (9%)	1 (25%)	0 (0%)	
Ex-smoker	13 (12%)	12 (13%)	1 (25%)	0 (0%)	
Not known	54 (50%)	47 (51%)	1 (25%)	6 (60%)	
SCC antigen					
Normal ($<2.0\,\mu g\,l^{-1}$)	62 (58%)	51 (55%)	3 (75%)	8 (80%)	0.123
Elevated	38 (36%)	36 (39%)	1 (25%)	1 (10%)	
Not known	7 (7%)	6 (6%)	0 (0%)	1 (10%)	
Treatment					
IMRT only	16 (15%)	13 (14%)	0 (0%)	3 (30%)	—
5-FU + MMC + RT/IMRT	44 (41%)	37 (40%)	3 (75%)	4 (40%)	
CAP + MMC + IMRT	47 (44%)	43 (46%)	1 (25%)	3 (30%)	
Radiation dose					
Total radiation dose to primary tumour, median (range)	64.8 (59.4–70.2)	64.8 (59.4–70.2)	64.8 (59.4–68.4)	64.8 (59.4–64.8)	—
Total radiation dose to LNs, median (range)	54.9 (45.0–66.6)	54.9 (45.0–70.2)	54.9 (49.5–68.4)	54.9 (45.0–64.8)	—

Abbreviations: CAP = capecitabine; CF-RT = three-dimensional conformal radiotherapy; 5-FU = 5-fluorouracil; HPV = human papillomavirus; IMRT = intensity-modulated radiation therapy; LNs = lymph nodes; MMC = mitomycin C; SCC = squamous cell carcinoma; UICC = Union Internationale Contre le Cancer.
[a]Interquartile range for age was 51–66 years for HPV+/p16+, 45–69 years for HPV−/p16+, and 49–69 years for HPV−/p16−.
[b]Non-smoker *vs* smoker or ex-smoker, $P=1.000$; smoker *vs* non-smoker or ex-smoker, $P=0.686$).

with inferior LRC in univariate analysis (Figure 3). Within the group of HPV+/p16+ patients, p53 expression pattern did not predict for response ($P=0.280$ and $P=0.656$, not shown). Within the HPV− group, patients with aberrant p53 expression patterns tended to have inferior LRC ($P=0.095$) and inferior OS ($P=0.108$) compared with patients with non-aberrant expression (Supplementary Figure S3A and B). In contrast, outcome of patients with *TP53* mutations in the HPV− group was not significantly different from outcome of patients without detected *TP53* mutations in the same group ($P=0.349$ and $P=0.477$; Supplementary Figure S3C and D), suggesting that *TP53* mutational status *per se* was not a prognostic factor in HPV− tumours. The single patient with a disruptive *TP53* mutation in the HPV+ group achieved complete response and was disease-free at last follow-up 2.5 years after finishing treatment.

Multivariate analysis of prognostic factors. Subsequently, multivariate analyses of LRC and OS were performed by including covariates that were significant in univariate analysis. Table 4 summarises the results of these analyses. Human papillomavirus −/p16− status and male sex were both independently associated with reduced LRC. Furthermore, HPV−/p16− status as well as higher T-classification were independently prognostic for reduced OS.

DISCUSSION

In this large single institute cohort of patients with SCCAC given standard of care treatment, we show that HPV and p16 status are

Table 2. HPV types detected	
HR-HPV positive	**93 (87%)**
Single HR-HPV type	80 (75%)
HPV16	71
HPV18	4
HPV31	1
HPV33	1
HPV56	1
HPV82	1
Other HR type (not specified)	1
Combinations with HPV 16	10 (9%)
HPV16 + 52	4
HPV16 + 18	1
HPV16 + 33 + 66 + 6 + 43 + 74	1
HPV16 + 39/68 + 70	1
HPV16 + 11	1
HPV16 + 51	1
HPV16 + 6	1
Combinations of other HR-HPV	3 (3%)
HPV18 + 82 + 74	1
HPV31 + 44	1
HPV35 + 52	1
HR-HPV negative	**14 (13%)**
No HPV	**10 (9%)**
None detected	10
LR-HPV	4 (4%)
HPV6	4

Abbreviations: HPV = human papillomavirus; HR-HPV = high-risk HPV; LR-HPV = low-risk HPV.

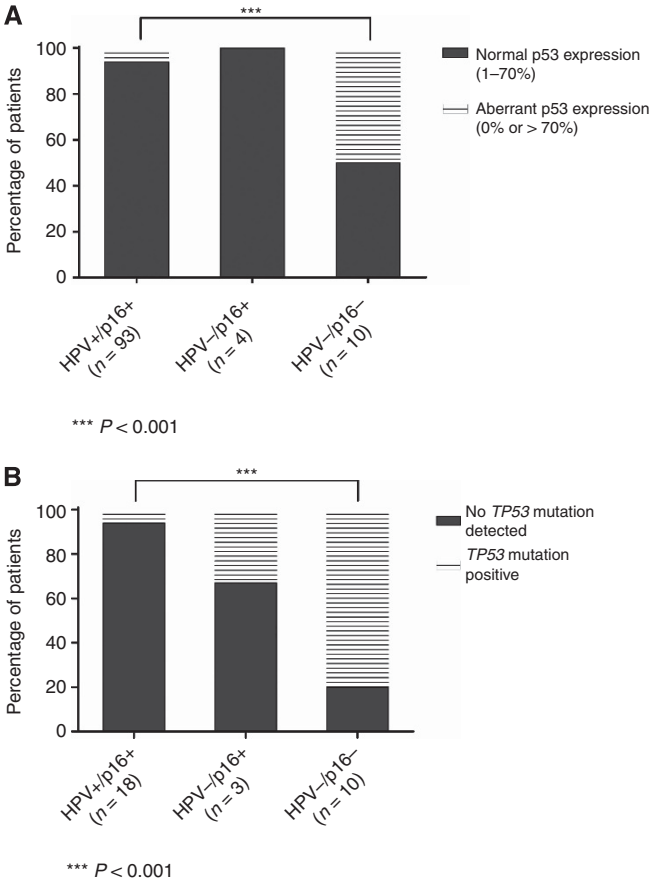

Figure 1. Expression patterns of p53 and disruptive *TP53* mutations according to HPV/p16 status. Distribution of p53 expression patterns (**A**) and disruptive *TP53* mutations (**B**) among groups according to HPV/p16 status.

strong predictors for LRC and OS. Outcome of patients with HPV − /p16 − tumours was considerably inferior compared with patients with HPV + /p16 + tumours, and HPV − /p16 − status was an independent prognostic factor for reduced LRC and OS. Disruptive alterations in *TP53* were frequently found in HPV − /p16 − tumours (80%), compared with only sporadically in the tested HPV + /p16 + tumours (6%).

We used a highly sensitive method to detect HPV DNA, which is considered the gold standard and is able to detect most HPV types. The distribution of HPV types, with HPV16 being the most prevalent HR-HPV type in HPV + tumours, is in accordance with previous studies investigating the prevalence of HPV in SCCAC (Daling *et al*, 2004; Hoots *et al*, 2009; Valmary-Degano *et al*, 2013). In our study, not all HPV − tumours were also p16 − . In fact, 4 out of 16 (25%) of the HPV − tumours showed overexpression of p16, to a similar extend as HPV + tumours. This is consistent with a previous study by Serup-Hansen *et al* (2014), who also showed that a proportion of HPV − SCCAC tumours was p16 positive. However, their finding was attributed to insufficient sensitivity of the HPV assay used. In the current study, a highly sensitive detection method was used and additional analyses were performed to preclude that these cases are false negatives. For these reasons, we believe it is plausible that these tumours are truly HPV − . Patients with HPV − /p16 + tumours differed from patients with HPV + /p16 + tumours, in that the former were exclusively males, whereas among HPV + /p16 + patients, there were slightly more women. There was a trend towards better LRC in these patients compared with HPV − /p16 − patients, but it concerned a very small number of patients and could well be a chance finding. It has been demonstrated in orophyrangeal squamous cell carcinoma (OSCC) also that a subset of HPV − tumours is p16 positive (Rietbergen *et al*, 2014). Rietbergen *et al* (2014) characterised a series of these discordant, HPV − /p16 + cases of OSCC, and found that patterns of loss-of-heterozygosity characterised these tumours as HPV − rather than HPV + . The mechanism of overexpression of p16 in these tumours, if truly HPV − , could be related to mutations in genes of other tumour suppressor proteins, including *RB1*. Our findings with respect to concordance between HPV and p16 status in SCCAC are in line with reports in HNSSC (Rietbergen *et al*, 2014), and suggest that p16 is not a perfect surrogate marker for tumour HPV status. Thus, although p16 IHC has high sensitivity, specificity might be insufficient, and the use of HPV PCR could still be required if SCCAC patients were to be stratified based on HPV status (e.g. for the purpose of future clinical studies). We showed that disrupted p53 function, measured as disruptive *TP53* mutations, was far more common in patients with HPV − /p16 − tumours than in HPV + /p16 + tumours. This inverse relationship between HPV + status and *TP53* mutations has also been demonstrated in HNSCC (Westra *et al*, 2008; Stransky *et al*, 2011), and might indicate, as is thought to be the case in HNSCC (Boyle *et al*, 1993; Nees *et al*, 1993), that disruption of p53 function is a key mechanism for HPV − SCCAC tumours to evolve. It is not surprising that mutations in *TP53* are found only sporadically in HPV + tumours, as the HPV oncoprotein E6 inhibits p53 function by targeting it for ubiquitination and degradation. An additional mutation in *TP53* would, therefore, not be required for these tumours to evolve. The apparent lower frequency of *TP53* mutations in HPV − /p16 + tumours could be explained by aberrations in other tumour suppressor proteins, which could be investigated in future studies. Concordance between p53 immunohistochemistry and *TP53* mutational analysis was low. This might explain why in most previous studies no association between

Table 3. Results from *TP53* mutational analysis (exons 2–11) according to HPV/p16 status

Patient	HPV/p16 status	Percentage p53 staining	*TP53* mutations detected	Exon	Effect on p53 protein	Functional consequence[a]
HPV + /p16 +						
1	HPV + /p16 +	20	—	—	—	—
2	HPV + /p16 +	60	c.592G > A	6	p.E198K	Functional
3	HPV + /p16 +	20	—	—	—	—
4	HPV + /p16 +	20	—	—	—	—
5	HPV + /p16 +	40	—	—	—	—
6	HPV + /p16 +	70	—	—	—	—
7	HPV + /p16 +	1	—[b]	—	—	—
8	HPV + /p16 +	1	c.455del1	5	p.P152fs	Non-functional
9	HPV + /p16 +	20	—	—	—	—
10	HPV + /p16 +	10	—	—	—	—
11	HPV + /p16 +	50	—	—	—	—
12	HPV + /p16 +	10	—	—	—	—
13	HPV + /p16 +	30	—	—	—	—
14	HPV + /p16 +	10	—	—	—	—
15	HPV + /p16 +	10	—	—	—	—
16	HPV + /p16 +	50	—	—	—	—
17	HPV + /p16 +	1	—	—	—	—
18	HPV + /p16 +	30	—	—	—	—
HPV − /p16 +						
19	HPV − /p16 +	20	—	—	—	—
20	HPV − /p16 +	10	—	—	—	—
21	HPV − /p16 +	60	c.844C > T	8	p.R282W	Non-functional
HPV − /p16 −						
22	HPV − /p16 −	90	c.466del1	5	p.R156fs	Non-functional
23	HPV − /p16 −	60	c.844C > T	8	p.R282W	Non-functional
24	HPV − /p16 −	50	—	—	—	—
25	HPV − /p16 −	0	c.801_802del	8	p.A268fs	Non-functional
26	HPV − /p16 −	20	c.770T > G	7	p.L257R	Non-functional
27	HPV − /p16 −	10	—	—	—	—
28	HPV − /p16 −	100	c.568C > A	6	p.P190T	Partially functional
29	HPV − /p16 −	95	c.844C > T	8	p.R282W	Non-functional
30	HPV − /p16 −	0	c.176_194dup	4	p.M66fs	Non-functional
			c.637C > T	6	p.R213X	
31	HPV − /p16 −	50	c.854A > T	8	p.E285V	Non-functional

Abbreviations: HPV = human papillomavirus; IARC = International Agency for Research on Cancer.
[a]Data from the IARC *TP53* database (Kato *et al*, 2003).
[b]No *TP53* mutations detected in exons 4–11, and exons 2 and 3 are not evaluable.

p53 overexpression and outcome has been found (Lampejo *et al*, 2010). Our results indicate that p53 immunohistochemistry is inadequate as a surrogate measure to detect *TP53* mutations in SCCAC. The current concept in HNSCC is that HPV + and HPV − tumours develop by at least two different pathways: one driven by exposure to oncoproteins expressed by HPV and the other by exposure to environmental carcinogens (such as alcohol and tobacco) without HPV involvement. Although it is unlikely that these same carcinogens are the main inducers of HPV − SCCAC, our study suggests that there might indeed be relevant differences in the genetic constitution of SCCAC tumours based on HPV status. From our results, and those of previous studies, it appears that male sex is associated with the development of HPV − SCCAC (Gilbert *et al*, 2013; Rödel *et al*, 2014; Serup-Hansen *et al*, 2014). Which other intrinsic and environmental factors are associated with HPV − SCCAC remains to be established. Loss of p53 function has been linked to resistance to radiotherapy (Lu and El-Deiry, 2009; Skinner *et al*, 2012; Kimple *et al*, 2013). It is therefore conceivable that patients with HPV − tumours have inferior treatment response owing to a higher frequency of disrupted p53 function (via *TP53* mutations). However, we did not find strong evidence to support this hypothesis, as there were no differences in outcome between patients with and without disruptive mutations in the subgroup of HPV − patients. These findings could either indicate that *TP53* mutations do not have an important role in treatment resistance of HPV − tumours or that additional (genetic) alterations in the subset of HPV − tumours contribute equally to treatment resistance (i.e. in tumours that do not carry *TP53* mutations). We identified sporadic *TP53* mutations in HPV + tumours, as has also been demonstrated in HNSCC (Lechner *et al*, 2013). It will be of interest to investigate which other genetic differences in HPV + and HPV − SCCAC tumours are related to outcome, as these differences might eventually provide an explanation for why HPV − tumours, and a subset of HPV + tumours, are not responsive to standard treatment.

A limitation of our study is that it is retrospective in nature, and the number of patients studied was relatively small. Notwithstanding this, HPV status as a prognostic factor in SCCAC has also been recognised by others, and several studies have now shown that patients with HPV − and/or p16 − disease have significantly worse outcome (Yhim *et al*, 2011; Gilbert *et al*, 2013; Rödel *et al*, 2014; Serup-Hansen *et al*, 2014). The prognostic significance of lack of p16 expression appears to be considerable, and it is striking that OS of patients with HPV − tumours appears to be reduced despite the fact that surgical salvage treatment is generally an effective treatment for most patients with locoregional relapse. We did not observe distinct patterns of relapse, but this should preferably be investigated in a larger population. Although it remains to be established why treatment resistance occurs, it is also unknown which could be effective treatment strategies for these patients. In a recent small study in patients with recurrent disease, the efficacy of docetaxel with cisplatin and 5-FU was described in patients with p16 +

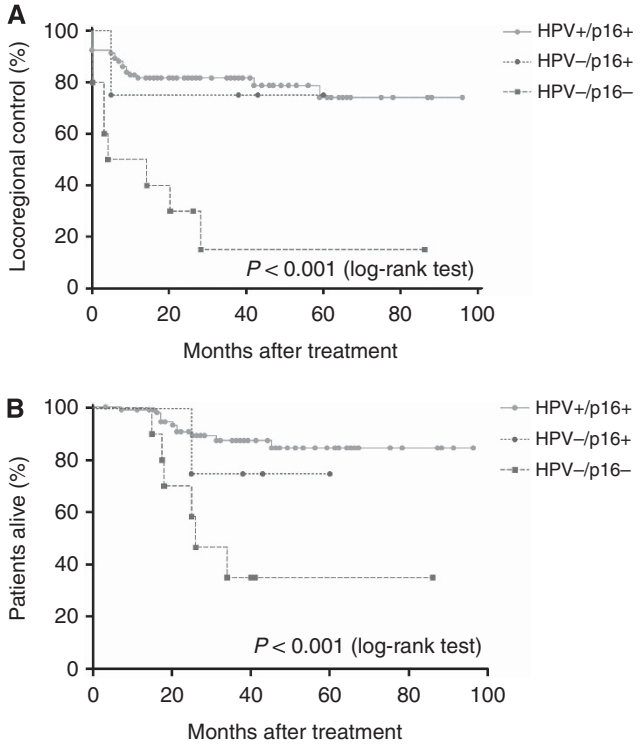

Figure 2. Outcome of patients according to HPV/p16 status. Locoregional control (**A**) and overall survival (**B**) of patients according to HPV/p16 status. The *P*-value represents an overall comparison of the three groups presented in the figure. Pairwise comparisons are given in the text.

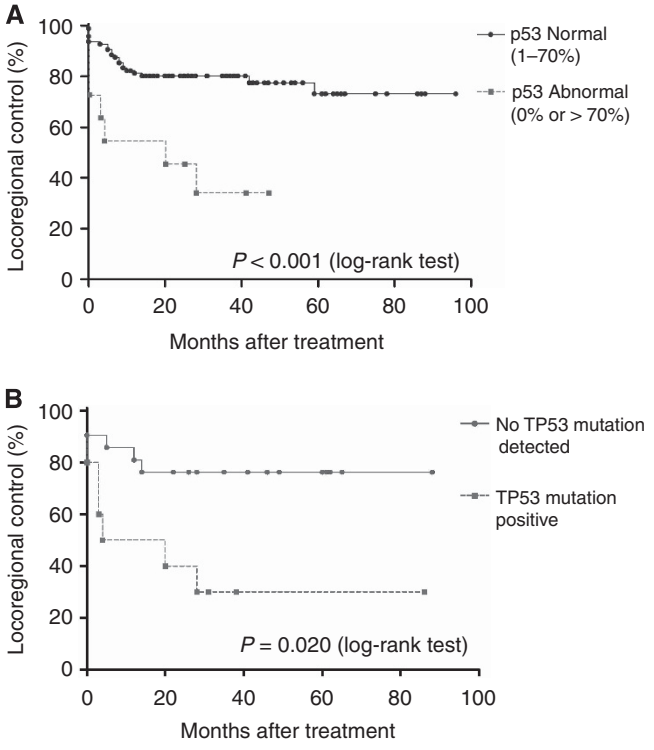

Figure 3. Outcome of patients according to p53 expression and *TP53* mutations. Locoregional control of patients according to p53 expression as measured by immunohistochemistry (**A**) and the presence of *TP53* mutations (**B**).

Table 4. Multivariate analysis of prognostic factors associated with locoregional control and overall survival

	Locoregional control				Overall survival			
	Univariate		Multivariate[a]		Univariate		Multivariate[a]	
Prognostic factor	**HR**	***P*-value**	**HR**	***P*-value**	**HR**	***P*-value**	**HR**	***P*-value**
HPV/p16 status[b]								
HPV + /p16 +	1.00		1.00		1.00		1.00	
HPV – /p16 +	1.19 (0.16–8.89)	0.867	0.75 (0.09–5.54)	0.753	1.10 (0.14–8.51)	0.929	0.95 (0.12–7.57)	0.961
HPV – /p16 –	5.59 (2.43–12.87)	<0.001	2.76 (1.01–7.56)	0.049	5.69 (2.09–15.50)	0.001	4.37 (1.59–12.07)	0.004
P53 expression								
P53 non-aberrant (1–70%)	1.00		1.00		1.00			
P53 aberrant (>70 or 0%)	4.07 (1.71–9.67)	0.002	2.39 (0.85–6.72)	0.100	2.71 (0.89–8.23)	0.080		
***TP53* mutations**								
None detected	1.00				1.00			
TP53 mutation positive	3.81 (1.20–12.08)	0.023			2.25 (0.85–6.00)	0.074		
Sex								
Female	1.00		1.00		1.00			
Male	3.45 (1.54–6.25)	0.003	3.07 (1.30–7.19)	0.010	2.25 (0.85–6.00)	0.105		
T-classification								
T1–2	1.00				1.00		1.00	
T3–4	1.44 (0.68–3.03)	0.341			4.52 (1.48–13.73)	0.008	4.03 (1.29–12.66)	0.017
N-classification								
N0–2	1.00				1.00			
N3–4	1.51	0.531			0.59 (0.08–4.43)	0.608		

Abbreviations:HPV = human papillomavirus; HR = hazard ratio.
[a]Variables that were significant in univariate analysis were analysed in the Cox model, except *TP53* mutations because of the limited number of samples analysed.
[b]HPV/p16 status was analysed as a factor with three levels.

and p16 – tumours. All patients with complete response had tumour p16 overexpression, whereas all patients with p16 – tumours were non-responders (Kim *et al*, 2013), suggesting that a full-dose chemotherapy regimen with a taxane and platinum in addition to a fluoropyrimidine might not be a valid approach for future studies in HPV – SCCAC. Concurrent

administration of the anti-EGFR monoclonal antibody cetuximab with chemoradiotherapy has been explored as a radiosensitising strategy, but has been shown to confer high rates of severe toxicity, prohibiting its integration in chemoradiotherapy regimens (Deutsch *et al*, 2013; Olivatto *et al*, 2013). In an experimental study, a role for cetuximab as maintenance treatment following radiotherapy has been suggested (Milas *et al*., 2007).

CONCLUSION

This study shows that, in addition to established prognostic factors such as T-classification and sex, outcome of patients with SCCAC is strongly determined by tumour HPV/p16 status. Determination of HPV and p16 may be useful clinically to predict patients' responsiveness to standard treatment, and in the context of clinical studies investigating novel treatment strategies for anal cancer, it might be crucial to stratify patients based on HPV status. We show that in contrast to HPV+ tumours, HPV− tumours frequently carry *TP53* mutations, suggesting that there might be large differences in the genetics of HPV+ *vs* HPV− tumours. Studies investigating these molecular characteristics are now required to develop effective treatment strategies for patients with treatment-resistant SCCAC.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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