Keywords: anal squamous cell carcinoma; mutation status; PIK3CA; prognostic factor; abdominoperineal resection; therapeutic target

Mutational analysis of anal cancers demonstrates frequent *PIK3CA* mutations associated with poor outcome after salvage abdominoperineal resection

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Background: A better understanding of the molecular profile of anal squamous cell carcinomas (ASCCs) is necessary to consider new therapeutic approaches, and the identification of prognostic and predictive factors for response to treatment.

Methods: We retrospectively analysed tumours from ASCC patients for mutational analysis of *KRAS*, *NRAS*, *HRAS*, *BRAF*, *PIK3CA*, *MET*, *TP53* and *FBXW7* genes by HRM and Sanger sequencing analysis.

Results: Specimens from 148 patients were analysed: 96 treatment-naïve tumours and 52 recurrences after initial radiotherapy (RT) or chemoradiotherapy (CRT). Mutations of *KRAS*, *PIK3CA*, *FBXW7* and *TP53* genes were present in 3 (2.0%), 30 (20.3%), 9 (6.1%) and 7 tumours (4.7%), respectively. The distribution of the mutations was similar between treatment-naïve tumours and recurrences, except for *TP53* mutations being more frequent in recurrences ($P=0.0005$). In patients treated with abdominoperineal resection (APR) after relapse ($n=38$, median follow-up of 18.2 years), overall survival (OS) was significantly correlated with HPV16 status ($P=0.048$), gender ($P=0.045$) and *PIK3CA* mutation ($P=0.037$). The *PIK3CA* status retained its prognostic significance in Cox multivariate regression analysis ($P=0.025$).

Conclusions: Our study identified *PIK3CA* mutation as an independent prognostic factor in patients who underwent APR for ASCC recurrence, suggesting a potential benefit from adjuvant treatment and the evaluation of targeted therapies with PI3K/Akt/mTor inhibitors in *PIK3CA*-mutated patients.

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Anal squamous cell carcinoma (ASCC) is a rare tumour that accounts for <5% of all lower gastrointestinal tract malignancies in Europe (Glynn-Jones *et al*, 2014). The incidence of ASCC has increased steadily in the past decades, particularly in women (Forman *et al*, 2012) and in men who have sex with men and those with HIV infection (Silverberg *et al*, 2012). Infection from human papilloma virus (HPV) is the main aetiological factor in the development of ASCC and >90% of patients are HPV positive (mainly HPV16 and 18) (Frisch *et al*, 1997; Abramowitz *et al*, 2011). Recent results of high-sensitivity HPV genotyping in a large series of ASCC patients showed a positivity rate of >95%. This supports the development of multivalent HPV vaccination for prevention (Baricevic *et al*, 2015). Concomitant chemoradiotherapy (CRT) is the standard of care for locally advanced tumours (Flam *et al*, 1996; Bartelink *et al*, 1997; Cacheux *et al*, 2012). So far, no predictive factor (to CRT) has been identified, excepted p16 expression, HPV status and *TP53* mutations (Gilbert *et al*, 2013; Koerber *et al*, 2014; Serup-Hansen *et al*, 2014; Baricevic *et al*, 2015; Mai *et al*, 2015; Meulendijks *et al*, 2015; Rödel *et al*, 2015). Salvage abdominoperineal resection (APR) is the standard treatment for local failure or recurrence after CRT, but 30 to 60% of operated patients will experience a locoregional and/or metastatic recurrence (Mullen *et al*, 2007; Mariani *et al*, 2008; Lefèvre *et al*, 2012; Correa *et al*, 2013). For these patients with an inoperable locally advanced or metastatic disease, very few treatments are available and their effectiveness is limited. New therapeutic approaches and predictive factors of outcome are required in this context. A better understanding of molecular markers involved in anal carcinogenesis might lead to the identification of new therapeutic targets as well as prognostic and predictive biomarkers. Recently, the potential effectiveness of anti-epidermal growth factor receptor (EGFR) monoclonal antibodies in advanced ASCC has been suggested by case reports (Lukan *et al*, 2009; Barmetter *et al*, 2012) that may be explained by both a high frequency of EGFR overexpression (80–90%) and the rarity of *KRAS* mutations in these tumours (Van Damme *et al*, 2010; Paliga *et al*, 2012; Smaglo *et al*, 2015). The incidence of other major gene alterations,

especially those implicated in the EGFR pathway, has been rarely studied in ASCC. In the present study, we examined the mutation status of *RAS* (*KRAS*, *NRAS* and *HRAS*), *BRAF*, *MET*, *FBXW7*, *TP53* and *PIK3CA* genes in a large series of 148 ASCC patients and correlated mutation status with clinicopathological characteristics and patient survival.

MATERIALS AND METHODS

Patient population. We retrospectively analysed tumours from ASCC patients consecutively treated from 1992 to 2015 at the Institut Curie Hospital. We included all consecutive patients for whom formalin-fixed, paraffin-embedded (FFPE) tumour tissue was available, and collected clinicopathological data and outcomes. This retrospective study was reviewed and approved by the Ethics Committee of the Institut Curie (No. A10-024). According to French regulations, patients were informed of research performed with the biological specimens obtained during their treatment and did not express opposition. Staging of the disease was based on the 7th revised edition (2010) of the AJCC Anus Cancer.

DNA extraction. Six tissue sections of 6 μ m thickness were obtained from FFPE tissues and a seventh tissue section stained with HE staining. The tumour-rich areas were microdissected using a single-use blade and the samples underwent proteinase K digestion in a rotating incubator at 56 °C for 3 days. DNA was extracted with the NucleoSpin kit (Macherey-Nalgen, Hoerd, France) according to the supplier recommendations in two separate aliquots that were analysed in parallel.

Gene mutation screening. The primer sequences used both for HRM and Sanger sequencing are shown in Supplementary Table 1. The majority of the HRM primers were designed to span the entire exons with product sizes under 200 bp. Primers were designed for *KRAS* (exons 2–4), *HRAS* (exons 2 and 3), *NRAS* (exons 2 and 3), *BRAF* (exon 15), *FBXW7* (exons 9 and 10), *PIK3CA* (exons 9 and 20), *MET* (exons 18 and 19) and *TP53* genes (exons 4–8) (Supplementary

Figure 1. Consort diagram of the study.

Table 1). The PCR for HRM and Sanger sequencing analysis was performed on a 384-well plate in the presence of the fluorescent DNA intercalating dye, LC green (Idaho Technology, Salt Lake City, UT, USA) in a LightCycler480 (Roche Diagnosis, Meylan, France). The reaction mixture in a 15 µl final volume contained LC green, UDP Glycosylase (Roche) and Roche Master Mix (Roche). The cycling and melting conditions were as follows: with an initial cycle of 10 min at 40 °C, one cycle of 95 °C for 10 min; 50 cycles of 95 °C for 10 s, 55–65 °C for 10 s, 72 °C for 30 s; one cycle of 97 °C for 1 min and a melt from 70 °C to 95 °C rising 0.2 °C per s. Depending on the melting temperature, a touchdown approach was done for some primers. All samples were tested in duplicate. The HRM data were analysed using the Genescan software (Roche). All samples including the wild-type exons were plotted according to their melting profiles on the differential plot graph. Any difference of the horizon line based on the wild-type sample was sequenced with Sanger sequencing.

Sanger sequencing. The reaction mixture in a total of 50 µl was made using 1 µl of PCR products without first purification followed by a sequencing reaction with Big Dye Terminator v3.1 (ThermoFisher, Courtaboeuf, France) according to the manufacturer's protocol. The sequencing products were purified with a Sephadex gel (GE Healthcare, Velizy-Villacoublay, France) before running on a 3500 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). The sequencing data were visualised using Finch TV (Geospiza, Inc., Seattle, WA, USA) with detection sensibility of 10% mutated cells.

HPV detection. From 1998 to 2013, all samples were analysed by PCR using specific primers to identify HPV16, 18, 33, 45, 6 and 11 types and using GP5+/GP6+ primers to detect HPV L1 DNA as previously described (Lombard *et al*, 1998). After 2013, real-time PCR using Sybr Green (Roche Diagnostics, Mannheim, Germany) and specific primers for HPV16, 18 and 33 and the human GAPDH gene was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems). HPV L1 amplicons from HPV16-, 18- and 33-negative samples were sequenced by Sanger method with GP6+ primer and HPV type identification was performed by alignment of the sequence with HPV sequence references, using the nucleotide blast program from NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Statistical analysis. The statistical analysis plan was predefined jointly by the authors. Overall survival (OS) was defined as the period from the first day of radiotherapy (RT) or CRT to death from any cause. Data on patients who were alive at the end of follow-up (November 2015) were regarded as censored. Progression-free survival (PFS) was defined as the period from the first day of RT or RCT to the date of first disease progression or death from any cause. Cox univariate and multivariate regression was used for survival analysis and Fisher's exact test was used for the analysis of contingency tables. All statistical tests were two sided and *P*-values of <0.05 were considered statistically significant. All analyses have been implemented in R version 3.2.1 R Development Core Team, 2013. The applicable R code can be found in the Supplementary Information.

RESULTS

Tumour and patient characteristics. A total of 148 ASCC samples from patients treated in our institution were included and analysed in our gene mutation screening as summarised in the consort diagram (Figure 1): 96 tumours were treatment naive and 52 were samples from recurrence after initial RT or CRT. In total, 142 tumours (95.9%) were HPV positive among which 131 tumours (88.5%) had HPV16 infection. Only 16 patients (10.8%) had HIV infection. In the HIV+ population (*n* = 16), all patients had concomitant HPV infection: 11 HPV16, 2 HPV18,

2 HPV6–11 and 1 HPV33. In the HIV – population (*n* = 132), 6 patients had no HPV infection and 126 had concomitant HPV infection: 120 HPV16, 1 HPV6–11, 1 HPV33, 2 HPV35, 1 HPV59 and 1 HPV67. Tumour characteristics according to the treatment-naïve or recurrence status of samples are summarised in Table 1.

There were 114 females and 34 males. The median age at the diagnosis was 61 years (range: 37–96 years). Twenty-five patients were treated by initial surgery: exclusive surgery (*n* = 5) and surgery followed by RT (*n* = 11) or RCT (*n* = 19). Thirty-two patients were treated by initial RT and 88 by initial CRT. One was treated by chemotherapy for an initial metastatic disease and 2 were not treated after the initial diagnosis. Only 16 patients (10.8%) had HIV infection. Forty-five patients underwent APR: 40 for local recurrence after RT or RCT, 3 at diagnosis and 2 for suspicion of local recurrence with complete histological response

Table 1. Clinicopathological features of treatment-naïve and recurrence tumour samples with subsequent treatment received (*n* = 148)

	Treatment-naïve tumour	Tumour recurrence
Total	<i>n</i> = 96	<i>n</i> = 52
Gender		
Female	77	37
Male	19	15
Site of tumour samples		
Anus	91	42
Lymph node	5	5
Liver	—	3
Other site	—	2
Concomitant HIV infection		
Yes	7	9
No	89	43
Tumour differentiation		
Poor	8	11
Moderate/well	88	41
HPV status		
HPV positive	95	47
Genotype 16	90	41
Other genotypes (6–11/33/35/67)	5 (1/1/2/1)	6 (2/2/1/1)
HPV negative	1	5
Prognostic groups (AJCC 2010)		
I	7	7
II	35	18
IIIA	21	6
IIIB	24	13
IV (liver/lymph node)	5 (4/1)	3 (2/1)
ND	4	5
Initial therapy^a		
Surgery (local excision/APR):	25	—
alone	5	—
followed by RT	11	—
followed by CRT	9	—
Radiation	11	21
Chemoradiation:	57	31
with concomitant 5FU-CDDP	47	26
with concomitant 5FU-MMC	5	3
with other concomitant CT	5	2
Chemotherapy	1	—
No treatment	2	—

Abbreviations: AJCC = American Joint Committee on Cancer; APR = abdominoperineal resection; CRT = chemoradiotherapy; CT = chemotherapy; HIV = human immunodeficiency virus; HPV = human papilloma virus; ND = not determined; RT = radiotherapy; 5FU-CDDP = 5-fluorouracil-cisplatin; 5FU-MMC = 5-fluorouracil-mitomycin C.

^aTreatment received after diagnostic tumour samples for treatment-naïve tumours and initial treatment received for tumour recurrence samples.

Table 2. (A) Prevalence of identified mutations in the 148 ASCC samples and distribution among treatment-naïve tumours and tumour recurrences. (B) Heterogeneity of mutational profiles in different tumour samples from the same patient (n = 3)								
(A)								
Genes	KRAS	HRAS	NRAS	BRAF	PIK3CA	MET	FBXW7	TP53
Total (%) (n = 148)	3 (2.0%)	0	0	0	30 (20.3%)	0	9 (6.1%)	7 (4.7%)
Exons	Exon 2: 3	—	—	—	Exon 9: 27 Exon 20: 3	—	Exon 9: 1 Exon 10: 8	Exon 4: 1 Exon 6: 1 Exon 7: 2 Exon 8: 3
Treatment-naïve samples (%) (n = 96)	1 (1.1%)	—	—	—	19 (19.8%)	—	4 (4.2%)	0 (0%)
Samples from recurrence (%) (n = 52)	2 (3.8%)	—	—	—	11 (21.2%)	—	5 (9.6%)	7 (13.5%)
P (Fisher’s test)	NS	—	—	—	NS	—	NS	0.0005
(B)								
	KRAS		PIK3CA		FBXW7			
Patient 1								
Recurrence (lymph node metastasis)	—		—		—			
Recurrence (peritoneal metastasis)	—		exon9:c.G1624A;p.E542K		exon10:c.1436G>A;p.R479Q			
Patient 2								
Treatment-naïve anal tumour	—		exon9:c.G1633A;p.E545K		—			
Anal recurrence	exon2:c.G34C;p.G12R		—		—			
Patient 3								
Treatment-naïve anal tumour	—		exon9: c.G1624A;p.E542K		—			
Treatment-naïve liver metastasis	exon2:c.G34C;p.G12R		exon9:.G1624A;p.E542K		—			
Abbreviations: ASCC = anal squamous cell carcinoma; NS = not significant.								

on surgical specimens. The median follow-up of 148 patients was 3.3 years (range: 0.2–39.6 years).

Gene mutation screening. Of the 148 tumours, 3 (2.0%) showed a *KRAS* exon 2 mutation, 30 (20.3%) a *PIK3CA* mutation, 9 (6.1%) a *FBXW7* mutation and 7 (4.7%) a *TP53* mutation (Table 2A and Supplementary Table 2). Five tumours (3.4%) had 2 synchronous mutations concerning these previous genes (*PIK3CA*/*FBXW7* mutations in 3 tumours, *KRAS*/*TP53* in 1 tumour and *FBXW7*/*TP53* in 1 tumour). All tumours were wild type for *HRAS*, *NRAS*, *BRAF* and *MET* genes. In 15 ASCC patients, we analysed several available samples obtained at different therapeutic times or in different sites. We observed a total concordance of the Sanger analysis in 12 patients but the mutational profile was different between samples for 3 patients (Table 2B).

Correlation between gene mutations and clinicopathological features and prognostic value. The distribution of the mutations was similar between treatment-naïve tumours and tumour recurrences, except for *TP53* mutations (Table 2A). We found that *TP53* mutations were restricted to recurrence samples: 7 of 52 (13.5%) tumour recurrences vs 0 of 96 (0%) treatment-naïve tumours (Fisher's test, *P* = 0.0005). Moreover, we observed that *TP53* mutations were more frequently associated with HPV16-negative samples: 3 of 131 (2.3%) HPV16-positive tumours vs 4 of 17 (23.5%) HPV16-negative tumours (Fisher's test, *P* = 0.003).

As the site and therapeutic status of tumour samples were heterogeneous in this large retrospective cohort of ASCC patients, we focussed our tumour analysis on homogenous groups of patients to study the association between mutational status and clinicopathological characteristics of the patients, and the impact of these parameters on OS. We also excluded nontreated tumours, tumours with ongoing treatment and those without sufficient follow-up (<6 months) in our prognostic analysis.

We identified a first group of treatment-naïve tumours from 57 ASCC patients treated by initial exclusive CRT with a median

follow-up of 3.1 years (range: 0.3–14 years) (Supplementary Table 3). Overall, recurrence rate was 24.6% (*n* = 14 of 57). All tumours were HPV positive and 52 of 57 (91.2%) had HPV type 16. Only 1 (1.7%) *KRAS*, 3 (5.3%) *FBXW7* and 1 (1.7%) *TP53* mutations were identified in this group, whereas *PIK3CA* mutations were identified in 10 (17.5%) of them (8 in exon 9 and 2 in exon 20). No association was found between *PIK3CA* mutations and clinicopathological characteristics of patients (data not shown). Moreover, no correlation was found between *PIK3CA* mutation and PFS or OS (Supplementary Table 4).

We also selected a second group of 40 recurrent tumour samples from ASCC patients who underwent APR for local recurrence after initial RT or CRT. We excluded 2 samples from patients who died early after APR from postoperative complications (at day 6 and 10 respectively). We obtained a final cohort of 38 ASCC samples with a median follow-up of 18.2 years (range: 0.82–39.6 years). Overall, recurrence rate was 57.9% (*n* = 22 of 38). Clinicopathological characteristics of this group of patients are summarised in Table 3. *PIK3CA*, *FBXW7* and *TP53* mutations were identified in 11 (28.9%), 5 (13.2%) and 4 (10.5%) recurrent tumours out of 38 respectively. No association was found between *PIK3CA* mutations and clinicopathological characteristics (Supplementary Table 5). A significant correlation by univariate Cox regression analysis was found between OS and gender (*P* = 0.045), HPV16 status (*P* = 0.048) and *PIK3CA* mutation (*P* = 0.037) (Table 4 and Figure 2). Multivariate Cox analysis showed that HPV16 status (*P* = 0.004), HIV status (*P* = 0.032) and *PIK3CA* mutation (*P* = 0.025) were independent prognostic factors (Table 4).

DISCUSSION

ASCC is known to be a very well radiosensitive tumour but 20% of patients failed to CRT, and no predictive markers of response have been prospectively validated. Moreover, in case of recurrence after RT/CRT, APR is the treatment of choice without any prognostic

Table 3. Clinicopathological features of the 38 tumour relapse samples from APR after initial RT/CRT

Total	n = 38
Gender	
Female	29
Male	9
Concomitant HIV infection	
Yes	7
No	31
HPV status	
HPV positive	34
genotype 16	29
other genotypes (6–11/18/33/59)	5 (1/2/1/1)
HPV negative	4
Initial therapy before APR	
Radiation	14
Chemoradiation with 5FU-CDDP	20
Chemoradiation with 5FU-MMC	3
Chemoradiation with other CT	1
Pathological results	
Tumour differentiation	
Poor	6
Moderate/well	32
ypT stage	
T0	1
T1	5
T2	19
T3	9
T4	3
ND	1
ypN stage	
N –	31
N +	7
Vascular emboli	
Yes	8
No	30
Lymphatic invasion	
Yes	9
No	29
Perineural invasion	
Yes	10
No	28
R1 resection	
Yes	8
No	30
PIK3CA mutation	
Yes	11
Exon 9	10
c.1624G > A;p.E542K	5
c.1633G > A;p.E545K	5
Exon 20	1
No	27
FBXW7 mutation	
Yes	5
No	33
TP53 mutation	
Yes	4
No	34

Abbreviations: APR = abdominoperineal resection; CRT = chemoradiotherapy; CT = chemotherapy; HIV = human immunodeficiency virus; HPV = human papilloma virus; ND = not determined; RT = radiotherapy; 5FU-CDDP = 5-fluorouracil-cisplatin; 5FU-MMC = 5-fluorouracil-mitomycin C.

Figure 2. Overall survival depending on the *PIK3CA* mutation in the 38 relapse APR patients after initial RT/RCT ($P = 0.025$).

Correa *et al*, 2013). In this context, a better biological and molecular characterisation of anal carcinogenesis is needed to improve the medical care of ASCC patients by identifying new therapeutic targets or prognostic biomarkers.

In the present study, which is the largest retrospective cohort of ASCC samples analysed by sequencing for multiple genes with complete clinicobiological data and long-term patient outcome available, we found frequent *PIK3CA* mutations (20.3%), as observed in previous smaller studies identifying *PIK3CA* mutation in 22% (11 out of 53, by pyrosequencing) and 32.5% (28 out of 86, by next-generation sequencing) of tumours (Casadei Gardini *et al*, 2014; Smaglo *et al*, 2015). The high level of *PIK3CA* mutation in ASCC provides a rationale to evaluate specific inhibitors of the *PIK3CA*/Akt/mTor pathway as demonstrated in preclinical models (Stelzer *et al*, 2010; Sun *et al*, 2013).

We identified very few *KRAS* exon 2 mutations (2.3%), in line with previous studies reporting low rates (Van Damme *et al*, 2010; Martin *et al*, 2014; Smaglo *et al*, 2015) or the absence of *KRAS* mutations (Paliga *et al*, 2012; Gilbert *et al*, 2013; Casadei Gardini *et al*, 2014) that could explain the effectiveness of EGFR monoclonal antibodies observed in ASCC patients (Lukan *et al*, 2009). The *TP53* mutations were also rarely described in the literature, although a high frequency of *TP53* protein expression is reported (Patel *et al*, 2007).

In our series, we confirm the low frequency of *TP53* mutations (5.3%) but we found they were restricted to recurrence samples and more frequently associated to HPV16-negative samples. As observed in our cohort, it was recently reported that *TP53* mutations were correlated with HPV16-negative status, predictive of resistance to RT/CRT and correlated with a poor prognosis in ASCC patients (Meulendijks *et al*, 2015). The same correlation between HPV status and *TP53* mutations was previously described in head and neck cancer (Westra *et al*, 2008).

We also focussed our gene screening on *FBXW7* gene. Mutations of *FBXW7* gene have been frequently reported in not only various squamous cell carcinomas (Agrawal *et al*, 2011; Gao *et al*, 2014; Ojesina *et al*, 2014) but also adenocarcinoma (The Cancer Genome Atlas Network, 2012; Laforest *et al*, 2014; The Cancer Genome Atlas Research Network, 2014) and melanoma where a protein inactivation was found (Aydin *et al*, 2014). For the first time, we report *FBXW7* mutations in 6% of ASCC. *FBXW7* is known to be a key regulator of the cell cycle involved in the maintenance of normal stem cells and cancer-initiating cells

factor identified or any adjuvant treatment recommendation, although at least 50% of patients experience recurrence after this surgery (Mullen *et al*, 2007; Mariani *et al*, 2008; Lefèvre *et al*, 2012;

Table 4. Overall survival according to clinicopathological and mutational characteristics of the 38 patients who underwent APR for tumour recurrence after RT/CRT

	Univariate analysis				Multivariate analysis			
	HR	95% Interval		P	HR	95% Interval		P
Gender (male)	3.034	1.026	8.968	0.045	—	—	—	NS ^a
ypT stage (T3–T4 vs T1–T2)	1.362	0.770	2.411	0.288				
ypN+ stage	2.302	0.792	6.700	0.126	3.108	0.861	11.211	0.083
R1 resection	2.398	0.770	7.463	0.131	—	—	—	NS ^a
Moderate/well tumoural differentiation	0.611	0.198	1.885	0.391				
Vascular emboli	1.392	0.450	4.309	0.566				
Lymphatic invasion	1.031	0.334	3.181	0.958				
Perineural invasion	1.063	0.373	3.034	0.909				
HPV16-positive status	0.344	0.119	0.990	0.048	0.155	0.043	0.558	0.004
HIV-positive status	2.700	0.866	8.390	0.087	4.259	1.130	16.054	0.032
Initial therapy (CRT vs RT)	1.430	0.525	3.895	0.482				
<i>PIK3CA</i> mutation	2.808	1.066	7.394	0.037	3.729	1.180	11.781	0.025
<i>FBXW7</i> mutation	0.668	0.151	2.950	0.595				
<i>TP53</i> mutation	0.590	0.133	2.609	0.486				

Abbreviations: APR = abdominoperineal resection; CRT = chemoradiotherapy; HIV = human immunodeficiency virus; HPV = human papilloma virus; HR = hazard ratio; NS = not significant; RT = radiotherapy. Univariate and multivariate Cox regression. Bold entries are used for significant P data values which are <0.05 (as explained in the statistical analysis).

^aContribution to the model was not significant after stepwise reduction.

(Takeishi and Nakayama, 2014). It could act as a critical tumour suppressor gene by targeting the NOTCH1 oncoprotein and therefore be an effective biomarker for the evaluation of Notch inhibitors in ASCC (Aydin *et al*, 2014). Our study finally shows that *HRAS*, *NRAS*, *BRAF* and *MET* genes are not mutated in ASCC.

Of note, we observed a different gene mutational status in 3 out of 15 patients for whom several tumour samples were available. For one of them, this could be the consequence of CRT on tumour DNA as the different mutational profiles were obtained from anal treatment-naïve and pretreated samples respectively. For the two remaining patients, we can make the assumption of tumour heterogeneity as the different mutational profiles were observed in samples from two different tumour sites.

Our study is the first one assessing the relation between clinicopathological characteristics and mutational status of several genes in a large series of ASCC patients with details on treatments received, allowing an exploratory assessment of gene mutation predictive and prognostic value. In the large biomarker analysis of 199 ASCCs recently reported by Smaglo *et al* (2015), only part of the tumours were the subject of a gene sequencing (8 to 86 according to the gene analysed) and almost no clinicopathological information was available, therefore avoiding any correlation to be performed. In another series of 103 ASCC patients, paraffin-embedded tumour tissue was sufficient to perform analysis of *KRAS*, *BRAF* and *PIK3CA* gene mutation in only 50 patients (Casadei Gardini *et al*, 2014). In our study, none of the gene mutations identified was associated with clinicopathological characteristics. Casadei Gardini *et al* (2014) also found no association between *PIK3CA* mutations (found in 22% of cases) and clinical characteristics.

Several studies have reported on potential prognostic and predictive biomarkers of response to RT/CRT in ASCC (Lampejo *et al*, 2010; Myklebust *et al*, 2012; Fraunholz *et al*, 2013) but none of them was a gene mutation and none has been sufficiently validated to be used in clinical practice. In our large cohort, we selected 2 homogenous groups of patients regarding the treatments received to analyse: (1) the prognostic and predictive value on response to treatment of identified mutations in treatment-naïve samples of patients exclusively treated by CRT ($n = 57$) and (2) the

prognostic value of identified mutations after APR for local recurrence following RT/CRT ($n = 38$).

In the naïve tumours treated by CRT, *PIK3CA* mutation identified on pretreatment samples was not found prognostic or predictive of response to CRT. This result is concordant with the study of Casadei Gardini *et al* (2014) in which *PIK3CA* mutation was not associated with PFS or OS of patients treated by CRT. The predictive impact of this mutation on tumour response to CRT was not explored in this study (Casadei Gardini *et al*, 2014). We could not study the prognostic or predictive value of *KRAS*, *FBXW7* and *TP53* mutations given their low frequency in our study.

To our knowledge, this is the first study assessing gene mutations as potential prognostic biomarkers in ASCC patients who underwent APR for local recurrence after RT or RCT. After multivariate Cox analysis we identified three independent factors associated with worse survival: a negative HPV16 status ($P = 0.004$) and a positive HIV infection ($P = 0.032$), which has already been reported (Wexler *et al*, 2008; Yhim *et al*, 2011), and also the presence of *PIK3CA* gene mutation ($P = 0.025$) that is identified for the first time as a new independent prognostic marker in this setting. Of course, the prognosis value of *PIK3CA* mutations we report need to be validated in an independent and larger prospective cohort of ASCC, considering the relatively small sample size of our series. These *PIK3CA* mutations have been previously reported to be associated with poor prognostic in colorectal cancer (Barault *et al*, 2008; Ogino *et al*, 2009) but data in cervical squamous cell carcinoma are more divergent with both an association with better OS in early tumour stages (McIntyre *et al*, 2013) and a poor response following standard CRT in more advanced stages (de la Rochefordiere *et al*, 2015). Finally, gynecological cancer patients with *PIK3CA* mutations are more responsive to PI3K/Akt/mTor inhibitors than nonmutated patients (Husseinizadeh and Husseinizadeh, 2014). These results, together with our findings, suggest that *PIK3CA* mutations might play a major role in HPV-related squamous cell carcinoma, including anal carcinogenesis, especially in mechanisms of resistance to RT or CRT. They provide a rationale for the use of PI3K/Akt/mTor pathway inhibitors in radioresistant tumours, particularly in adjuvant setting after APR. Aspirin therapy, recently shown to be of particular efficacy in adjuvant treatment of *PIK3CA*-mutated

colorectal cancer, could be another therapeutic option in this setting (Liao *et al*, 2012). In addition, there are recent data suggesting that the host immune reaction mediates response (Gilbert *et al*, 2016) via tumour-infiltrating lymphocytes. These data suggest the evaluation of immunotherapy in anal cancer, whose efficiency might be enhanced by cyclooxygenase inhibitors such as aspirin (Zelaney *et al*, 2015).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors contributed to the conception and design of the study, acquisition of data, analysis and interpretation of data, drafting the article or revising it critically for important intellectual content and final approval of the version to be published.

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