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-naive 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-naive 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 (Glynne-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 aetiologic 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 μm 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, Hoerdt, 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

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**Figure 1. Consort diagram of the study.**
From 1998 to 2013, all samples were analysed by HPV detection. In total, 142 tumours (95.9%) were HPV positive among which 52 were samples from recurrence after initial RT or CRT. Two-forty-five patients were treated by initial surgery: exclusive surgery (n = 5) and surgery followed by RT (n = 11) or CRT (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 CRT, 3 at diagnosis and 2 for suspicion of local recurrence with complete histological response.

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

| Treatment-naive tumour | Tumour recurrence |
|------------------------|-------------------|
| Total                  | n = 96            |
|                       | n = 52            |
| Gender                 |                   |
| Female                 | 77                |
| Male                   | 19                |
|                       | 37                |
|                       | 15                |
| Site of tumour samples |                   |
| Anus                   | 91                |
| Lymph node             | 5                 |
| Liver                  | —                 |
| Other site             | —                 |
|                       | 42                |
|                       | 5                 |
|                       | —                 |
|                       | 2                 |
| Concomitant HIV infection |               |
| Yes                    | 7                 |
| No                     | 89                |
|                       | 9                 |
|                       | 43                |
| Tumour differentiation |                   |
| Poor                   | 8                 |
| Moderate/well          | 88                |
|                       | 11                |
|                       | 41                |
| HPV status             |                   |
| HPV positive           | 95                |
| Genotype 16            | 90                |
| Other genotypes (6–11/33/35/67) | 5 (1/1/2/1) |
| HPV negative           | 1                 |
|                       | 47                |
|                       | 41                |
|                       | 6 (2/2/1/1)       |
|                       | 5                 |
| Prognostic groups (AJCC 2010) |               |
| II                     | 7                 |
| IIIA                   | 35                |
| IIIB                   | 21                |
| IV (liver/lymph node)  | 24                |
| ND                     | 5                 |
|                       | 7                 |
|                       | 13                |
|                       | 3 (2/1)           |
|                       | 4                 |
|                       | 5                 |
| Initial therapy*       |                   |
| Surgery (local excision/APR): |             |
| alone                  | 25                |
| followed by RT         | 5                 |
| followed by CRT        | 11                |
| Radiation              | 9                 |
| Chemoradiation:        | 11                |
| with concomitant SFU-DDP | 26               |
| with concomitant SFU-MMC | 5                 |
| with other concomitant CT | 3               |
| Chemotherapy           | 5                 |
| 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, SFU- CDDP = 5-fluorouracil-cisplatin, SFU-MMC = 5-fluorouracil-mitomycin C. *Treatment received after diagnostic tumour samples for treatment-naive tumours and initial treatment received for tumour recurrence samples.

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-naive or recurrence status of samples are summarised in Table 1.
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-naive 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-naive 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-naive 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 PI3KCA 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: 8.2–39.6 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
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; 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 known to be a key regulator of the cell cycle involved in the maintenance of normal stem cells and cancer-initiating cells.
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, gynaecological cancer patients with PIK3CA mutations are more responsive to PI3K/Akt/mTor inhibitors than nonmutated patients (Husseinzadeh and Husseinzadeh, 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
Prognostic value of PIK3CA mutation in anal cancer

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