Pathogenic alterations in PIK3CA and KMT2C are frequent and independent prognostic factors in anal squamous cell carcinoma treated with salvage abdominoperineal resection

Abderaouf Hamza, Julien Masliah-Planchon, Cindy Neuzillet, Jérémie H Lefèvre, Magali Svrcek, Sophie Vacher, Christine Bourneix, Matthieu Delaye, Diane Goéré, Peggy Dartigues, et al.

Title page

Title

Pathogenic Alterations in *PIK3CA* and *KMT2C* are Frequent and Independent Prognostic Factors in Anal Squamous Cell Carcinoma Treated with Salvage Abdominoperineal Resection

Short title

Genomic biomarkers in anal squamous cell carcinomas

Authors

Abderaouf Hamza[1], Julien Masliah-Planchon[1], Cindy Neuzillet[2], Jérémie H. Lefèvre[3], Magali Svrcek[4], Sophie Vacher[1], Christine Bourneix[1], Matthieu Delaye[2], Diane Goéré[5], Peggy Dartigues[6], Emmanuelle Samalin[7], Marc Hilmi[2], Julien Lazartigues[2], Elodie Girard[8], Jean-François Emile[9], Eugénie Rigault[10], Virginie Dangles-Marie[11,12], Nathalie Rioux-Leclercq[13], Christelle de La Fouchardière[13], David Tougeron[14], Andrea Casadei-Gardini[15], Pascale Mariani[16], Frédérique Peschaud[17], Wulfran Cacheux[18], Astrid Lièvre[10,19,*], Ivan Bièche[1,20,*]

Affiliations

[1] Genetics Department, Institut Curie, PSL Research University, Paris, France

[2] Medical Oncology Department, Institut Curie, PSL Research University, Saint-Cloud, France

[3] Sorbonne Université, Department of Digestive Surgery, Assistance Publique-Hôpitaux de Paris, Hôpital Saint Antoine, F-75012, Paris, France.

[4] Pathology Department, Hôpital Saint-Antoine, Assistance Publique-Hôpitaux de Paris, France.

[5] Digestive Surgery Department, Gustave Roussy Institute, Villejuif, France.

[6] Pathology Department, Gustave Roussy Institute, Villejuif, France.

[7] Medical Oncology Department, Institut du Cancer de Montpellier, Montpellier, France.

[8] INSERM U900 Research unit, Institut Curie, PSL Research University, Paris, France.

[9] Université Paris-Saclay, Assistance Publique-Hôpitaux de Paris, UVSQ, BECCOH, Hôpital Ambroise-Paré, Pathology Department, Boulogne-Billancourt, France.

[10] Gastroenterology Department, Rennes University Hospital, Rennes, France.

[11] Laboratory of preclinical investigation, Translational Research Department, Institut Curie, PSL Research University, Paris, France.

[12] Faculty of Pharmaceutical and Biological Sciences, Paris Cité University, Paris, France.

[13] Medical Oncology Department, Centre Léon Bérard, Lyon, France.

[14] Department of Gastroenterology and Hepatology, Poitiers University Hospital, Poitiers, France.

[15] Department of Oncology, Vita-Salute San Raffaele University, IRCCS San Raffaele Scientific Institute Hospital, Milan, Italy.

[16] Department of Surgery, Institut Curie, PSL Research University, Paris, France.

[17] Department of Digestive and Oncologic Surgery, Ambroise Paré Hospital, Versailles Saint-Quentin University, Paris Saclay University, Boulogne-Billancourt, France.

[18] Medical Oncology Department, Hôpital Privé Pays de Savoie, Annemasse, France.

[19] Rennes 1 University; Inserm U1242, COSS (Chemistry Oncogenesis Stress Signaling), Rennes, France.

[20] INSERM U1016, Faculty of Pharmaceutical and Biological Sciences, Paris Cité University, Paris, France.

[*] These authors contributed equally to this work.

Abbreviations

APR: abdominoperineal resection

ASCC: anal squamous cell carcinoma
CNV: copy number variant
CRT: chemoradiotherapy
CT: chemotherapy
DFS: disease-free survival
FFPE: formalin-fixed paraffin-embedded
indel: insertion and deletion
IQR: inter-quartile range
MFS: metastasis-free survival
NGS: next-generation sequencing
OS: overall survival
RT: radiotherapy
SNV: single nucleotide variant

Correspondence

Prof. Ivan Bièche
ivan.bieche@curie.fr
+33172389363
Service de Génétique, Institut Curie, PSL Research University, 26 rue d'Ulm, 75005 Paris, France.

Key words

Next-generation sequencing; precision medicine; gastrointestinal cancer; genomic biomarkers; HPV associated cancer

Word count

3313

Novelty and Impact

The genomics of anal squamous cell carcinomas has been poorly studied, depriving patients of the benefits of precision medicine. We constituted a large retrospective cohort of cases of this

rare disease to uncover molecular prognostic and theragnostic biomarkers. We show that *PIK3CA* and *KMT2C* pathogenic variants have prognostic values comparable to that of established clinical factors, and that more than 40% of patients have tumors with potentially targetable mutations. These findings support systematic molecular profiling and inclusion of anal squamous cell carcinomas patients in precision medicine trials.

Abstract

The management of anal squamous cell carcinoma (ASCC) has yet to experience the transformative impact of precision medicine. Conducting genomic analyses may uncover novel prognostic biomarkers and offer potential directions for the development of targeted therapies. To that end, we assessed the prognostic and theragnostic implications of pathogenic variants identified in 571 cancer-related genes from surgical samples collected from a homogeneous, multicentric French cohort of 158 ASCC patients who underwent abdominoperineal resection treatment. Alterations in PI3K/AKT/mTOR, chromatin remodeling, and Notch pathways were frequent in HPV-positive tumors, while HPV-negative tumors often harbored variants in cell cycle regulation and genome integrity maintenance genes (e.g., frequent *TP53* and *TERT* promoter mutations). In patients with HPV-positive tumors, *KMT2C* and *PIK3CA* exon 9/20 pathogenic variants were associated with worse overall survival in multivariate analysis (Hazard ratio $(HR)_{KMT2C}$ = 2.54, 95%CI = [1.25, 5.17], p-value = 0.010; HR_{PIK3CA} = 2.43, 95%CI = [1.3, 4.56], p-value = 0.006). Alterations with theragnostic value in another cancer type was detected in 43% of patients. These results suggest that *PIK3CA* and *KMT2C* pathogenic variants are independent prognostic factors in patients with ASCC with HPV-positive tumors treated by abdominoperineal resection. And, importantly, the high prevalence of alterations bearing potential theragnostic value strongly supports the use of genomic profiling to allow patient enrollment in precision medicine clinical trials.

Keywords

Genomic biomarker; gastrointestinal cancer; precision medicine; cancer genomics

Text

Introduction

Anal canal cancer is a rare yet increasingly prevalent disease, with incidence rates dramatically rising over the last two decades: in the United States, Nelson et al. reported a 7.2% annual percentage change in anal cancer incidence between 1997 and 2009 (1). Anal squamous cell carcinoma (ASCC), the most common histological subtype, originates in the anal mucosa between the rectum and anal verge (2). It is associated with HPV infection in 90-95% of cases (3), while other risk factors include immune suppression (4), sexual behavior (5) and tobacco smoking (6,7).

The primary treatment goal for most patients with local/locoregional anal canal cancer is curative while preserving anal sphincter function. Since the 1970s, the standard of care has been chemoradiotherapy (CRT), combining radiotherapy (RT) with 5-fluorouracil-based chemotherapy (CT)(8,9). This regimen achieves complete pathological response in roughly 80% of patients but is linked to significant toxicity (10,11). Salvage abdominoperineal resection (APR) is considered for primary RT/CRT failure or locoregional relapse cases, but is associated with high morbidity, reduced quality of life, and poor long-term survival (12). Metastatic cases receive first-line carboplatin and paclitaxel-based systemic chemotherapy, and while immunotherapy with PD-1/PDL-1 inhibitors is being explored, current guidelines limit its use to second-line in metastatic settings (8,9). No targeted therapy has been approved for ASCC treatment to date.

The rarity of ASCC presents challenges in investigating potential prognostic factors. Although the influence of sex and other clinical or histopathological characteristics remains debated,

tumor size and lymph node involvement are generally regarded as reliable predictors of outcome (13). However, these factors are insufficient for predicting CRT failure and relapse. Recent studies have shown that the absence of HPV infection is a negative prognostic factor (14–16), but no other molecular biomarker has emerged.

Compared to other cancers, the genomic features of ASCC, their association with clinical outcomes, and their potential theragnostic value have been poorly studied. Notably, ASCC was excluded from The Cancer Genome Atlas program, a comprehensive pan-cancer molecular characterization initiative (17). Previous reports (18–23) describe genomic features typical of squamous cancers (24) like frequent PI3K/AKT/mTOR pathway activation, Notch paralogs alterations, and few, if any, RAS/RAF-protein mutations. But, due to the rarity of the disease, most of the cohorts described are small, heterogeneous, or poorly characterized, and the few proposed association between genomic features and clinical outcomes have not been replicated.

Improving ASCC patient management may be achieved by identifying novel prognostic and theragnostic genomic biomarkers in those unresponsive to first-line interventions. This retrospective longitudinal multicentric study presents an extensive genomic profile of a large, homogeneous, and well-characterized cohort of ASCC patients treated with APR following RT/CRT failure. The study aims to evaluate the prognostic value of genomic biomarkers and identify theragnostic biomarkers to explore potential avenues for targeted therapy.

Materials and Methods

Case selection and data collection

This retrospective multicentric study included all eligible ASCC patients who underwent APR in nine French medical centers from January 1996 to February 2016. Indications of APR were tumor persistence (*i.e.*, persistent ulceration or re-emergence of the anal lesion within six

months of treatment completion) or recurrence (*i.e.*, lesions appearing more than six months after treatment completion) after first-line RT or CRT. Inclusion criteria were availability of formalin-fixed paraffin-embedded (FFPE) tissue sample obtained from APR, histologically confirmed squamous cell carcinoma, availability of clinical and histopathological records, and a follow-up period of at least two years. Clinical and histopathological data collected from medical records included age at APR, sex, indication for surgery, disease staging at the time of APR per the American Joint Committee on Cancer (AJCC, 8th edition), degree of differentiation, presence of vascular emboli, type of pre-operative treatment, HIV-status, tumor HPV-status, recurrence, relapse and metastasis data, and survival status.

Genomic DNA extraction

For each patient in this study, seven tissue sections of 6 µm thickness were obtained from a single FFPE tissue block. Tumor-rich areas were identified on one hematoxylin-eosin-stained slide and microdissected using a single-use blade on the remaining six slides for extraction. Samples underwent proteinase K digestion at 56 °C for 3 days, followed by DNA extraction using the NucleoSpin kit (Macherey-Nagel, Düren, Germany) according to the supplier's protocols. DNA was quantified using a Qubit dsDNA HS assay kit (ThermoScientific, Wilmington, DE, USA).

Next-Generation Sequencing and bioinformatic analysis

The genomic profiling of tumor samples was conducted on the DRAGON Dx (Detection of Relevant Alterations in Genes involved in Oncogenetics) platform, a pan-cancer diagnostic next-generation sequencing (NGS) panel comprising 571 genes (25) (Supplementary Table 1). The assay allows the detection of single nucleotide variants (SNV), small (<50bp) insertions and deletions (indels), copy number variants (CNV), and microsatellites instability. Library preparation, sequencing, and bioinformatic analysis were performed as described previously

(25). Quality-control criteria for sequencing data were: at least 1 million reads mapped, and at least 5% of targeted regions at a sequencing depth of 1000X or more. The sequencing coverage and quality statistics for each sample are summarized in Supplementary Table S2.

Downstream analysis and clinical annotation

SNV and indels

Only variants with a depth of coverage of at least 100X and allelic frequency of at least 10% were considered for downstream analysis. Strand bias was quantified by the Phred-scaled *p*-value of the Fisher's exact test on the contingency table of the strand-specific distribution of reads supporting either the reference or alternative allele. SNV and indels with a score higher than 50 and 200, respectively, were filtered out. Variants with reported frequencies higher than 0.5% in any sub-population in either gnomAD(26) (version 2.1.1) or Exome Sequencing Project (version ESP6500) databases were excluded from the analysis. MSISensor2 was used to detect microsatellite instability(27).

Copy number variants

Gain and shallow deletions were defined as DNA segments with a log2 median ratio higher than 0.5 or lower than -0.5, respectively; amplifications and homozygous deletions were defined as DNA segments <10Mb with a log2 median ratio higher than 2 or lower than -1.5, respectively. Only amplification and homozygous deletions were considered for functional and theragnostic annotation.

Functional and theragnostic annotation

Genes were classified as tumor suppressors, oncogenes, or both, and grouped in cellular pathways according to the literature and authoritative databases (cBioPortal (28), OncoKB (29), The Cancer Genome Atlas (30), COSMIC (31)); genes with equivocal evidence for their specific role in carcinogenesis where classified as unknown (Supplementary Table 1). Splice mutations in tumor suppressor genes, stop-gain, and frame-shift variants were grouped under the "truncating variants" class. Variant pathogenicity and theragnostic significance were assessed using automated analysis using the OncoKB-annotator tool (32) and manual curation. Were classified as pathogenic: all variants labeled as oncogenic, likely oncogenic, or predicted oncogenic by the OncoKB-annotator tool; all truncating variants in tumor suppressor genes; and variants with sufficient evidence for their oncogenic effect in the literature and databases in oncogenes and tumor suppressor genes (e.g., hotspot missense variants).

Statistical analysis

As appropriate, distributions of categorical variables and continuous variables between groups were compared with Fisher's exact test or Student's t-test; p-values were adjusted for multiple testing using the Benjamini and Hochberg method. Mutual exclusion or cooccurrence of alteration were assessed with the DISCOVER R package (33). Survival endpoints were defined in accordance with the DATECAN consensus (34): overall survival (OS) was defined as the time between APR and death from any cause, disease-free survival (DFS) as the time between APR and relapse (whether local or distant) of any type or death, and metastasis-free survival (MFS) as the time between APR and metastatic relapse or death. Patients were censored at the date of last follow-up if no event was recorded. Only genes found altered in more than 5% of samples were included in survival analyses. Multivariate Cox proportional hazard models were built following a two-step process: first, candidate genes with significant association with survival in univariate analyses (log-rank test p-value < 0.1) were included in a multivariate Cox model with clinical and histopathological variables of known relevance (sex, age, TNM staging

including resection margin status, degree of differentiation). Then, after testing for redundancy and proportional hazard assumption validity, candidate genes were selected through a stepwise method minimizing the Akaike criterion. Survival curves for genes significantly associated with survival were drawn using the Kaplan–Meier method and compared with the log-rank test. All statistical analyses were performed using the R statistical language. Univariate and multivariate Cox models were computed with the survival package (version 3.2-11); Kaplan-Meier curves were computed and drawn using the survminer package (version 0.4.9); forest plots were drawn using the forestmodel package (version 0.6.2).

Results

Cohort characteristics

Out of 177 patients screened, 160 met the inclusion criteria, and sequencing quality control criteria were met for 158 samples (Table 1). The median age at APR was 57 (IQR = [49,66]). Most samples were HPV-positive (141/158, 89%), primarily with HPV-16 (126/141, 89%) and HPV-18 (5/141, 4%) serotypes. Most patients were female (102/158, 65%); however, males were overrepresented in the HPV-negative group (12/17, 71% vs. 44/141, 31%, p-value = 0.001). Although the most prevalent indication for surgery across all cases was tumor recurrence (115/158, 73%), the proportion of patients referred to surgical treatment for tumor persistence was higher in the HPV-negative group than in the HPV positive group (10/17, 59%, vs. 33/141, 23%, p-value = 0.004). Surgical specimens mostly consisted of lymph node-negative tumors (125/156, 80%), with moderate/high differentiation (120/156, 77%), and R0 resection margins (123/157, 79%). Chemoradiotherapy was the predominant pre-operative treatment in the HPV-positive group, but not in the HPV-negative group (104/156, 74% vs. 7/17, 44%, p-value = 0.018).

Genomic profiling

For samples meeting sequencing quality control criteria, sequencing metrics were consistent with sensitive variant detection across the entire gene panel. The median number of detected variants per sample, including amplifications and homozygous deletions, was 52 (IQR = [43, 80]). The median number of pathogenic variants detected per sample was 2 (IQR = [1, 4]), with one or more pathogenic variants found in 88% (137/158) of samples (Figure 1). Microsatellite instability was not detected in any of the 158 samples studied.

Mutational profiles notably differed between HPV-positive and HPV-negative patients (Figure 1, Supplementary Table 3, and Supplementary Table 4). Among HPV-positive samples, the most frequently altered genes (i.e., found in at least 10% of samples) were *PIK3CA*, *KMT2D*, *KMT2C*, *FBXW7*, and *FAT1*, collectively altered in 52% (75/141) of cases. In contrast, among HPV-negative samples, the most frequently altered genes were *TP53*, *TERT*, *CDKN2A*, *ARID1A*, *NFE2L2*, *FAT1*, and *KMT2C*, collectively found in 82% (14/17) of cases. *TERT* mutations (ten c.-124C>T and one c.-146C>T promoter region variants, and one amplification) were significantly more frequent in HPV-negative samples (4/141, 3% vs. 8/17, 47%, respectively, adjusted p-value = 1.20•10-6), as were alterations in *TP53* (7/141, 5% vs. 10/17, 59%, adjusted p-value = 1.38•10-7).

Some additional alterations exhibited a skewed distribution between the two viral status groups, but the difference did not reach statistical significance. *CDKN2A* alterations, all loss-of-function missense or truncating mutations, were more frequent in HPV-negative samples (1/141, 1%, vs. 3/17, 18%). *PIK3CA* variants were disproportionately found in HPV-positive samples (39/141, 28% vs. 1/17, 6%); the most frequent were exon 9 variants (c.1633G>A/p.(E545K), 18/40, 45%; c.1624G>A/p.(E542K), 10/40, 25%), followed by exon 20 variants (c.3140A>G/p.(H1047R), 2/40, 5%; c.3129G>C/p.(M1043I) 1/40, 3%). All but

two of the 48 loss-of-function variants in *KMT2*-family genes (KMT2D, n = 23; KMT2C, n = 22; KMT2B, n = 3) were found in HPV-positive tumors. All 19 *FBXW7* pathogenic variants, including one homozygous deletion, were found exclusively in HPV-positive tumors; 84% (15/19) of them were hotspot loss-of-function missense mutations affecting the substrate-binding domain (tryptophan-aspartic acid motif, WD40). *PIK3CA* and *EP300* alterations were mutually exclusive in the HPV-positive group (adjusted p-value = 0.04) (Supplementary Figure 1); no other significant co-occurrence or mutual exclusion pattern was detected. Copy number profiles also showed notable differences depending on sample HPV status (Supplementary Figure 2). These differences paralleled mutation frequency disparities: 3q region gains, encompassing *PIK3CA* and *SOX2*, were more frequent in HPV-positive tumors than in HPV-negative tumors (64/141, 45% vs. 1/17, 6%, p-value = 0.001). Mutational profiles did not differ significantly between surgery indication groups.

When grouping genes by biological functions and cellular pathways, recurrently altered signaling pathways and cellular processes were revealed (Supplementary Figure 3 and Supplementary Table 5). PI3K/AKT/mTOR pathway gene alterations were significantly more frequent in HPV-positive tumors (60/141, 43% vs. 1/17, 6%, adjusted p-value = 0.04). Conversely, alterations in genes involved in genome integrity were significantly enriched in HPV-negative tumors (12/17, 71% vs. 15/141, 11%, adjusted p-value = 4.93•10-6). Pathogenic variations in Notch (32/141, 23% vs. 1/17, 6%) and TGF-ß pathways (12/141, 9% vs. 0/17, 0%) were found disproportionately in HPV-positive tumors, and cell cycle regulation genes (4/17, 24% vs. 14/141, 10%) were more frequent in HPV-negative tumors, although these differences did not reach statistical significance. Groups altered at similar frequencies in both viral status groups included genes involved in chromatin remodeling (67/141, 48% vs. 8/17, 47%), DNA

repair (38/141, 27% vs. 6/17; 35%), gene expression regulation (32/141, 23% vs. 4/17, 24%), RTK-Ras pathway (22/141, 16% vs. 3/17, 18%), and Hippo pathway (21/141, 15% vs. 2/17, 12%).

Survival analysis

Given the significant differences in genomic profiles and the unbalanced group sizes, survival analysis was conducted separately for the two viral status groups. Overall survival (OS) data were available for 134 of the 141 HPV-positive patients, while disease-free survival (DFS) and metastasis-free survival (MFS) data were available for 140 patients. For HPV-negative patients, OS data was available for 16 of the 17 patients, and DFS and MFS data were available for 17 patients. The median follow-up period was 63.6 months (interquartile range, IQR = [41.1,107.0]) for patients with HPV-positive tumors and 58.5 months (IQR = [26.1,73.9]) for patients with HPV-negative tumors. During the study period, there were 73 relapses (including 46 metastatic relapses) and 60 deaths in the HPV-positive group, while 8 relapses (including 4 metastatic relapses) and 6 deaths occurred in the HPV-negative group.

In univariate analyses, the association between pathogenic variants and survival outcomes was assessed for the ten genes found altered in more than 5% of HPV-positive cases (Supplementary Table 6). *KMT2C* pathogenic variants were negatively associated with OS (p-value = 0.005), DFS (p-value = 0.002), and MFS (p-value < 0.001). Exons 9/20 variants of PIK3CA were negatively associated with OS (p-value = 0.004), DFS (p-value = 0.044), and MFS (p-value = 0.006); no association with variants in other *PIK3CA* exons and survival outcomes was detected. In HPV-negative cases, the association between survival outcomes and the presence of a pathogenic variant was evaluated in univariate analysis for three genes (Supplementary Table 7). *TP53* alterations were significantly associated with shorter OS (p-

value = 0.041) and MFS (p-value = 0.016), while *TERT* alterations were significantly associated with shorter MFS (p-value = 0.033).

In multivariate analyses, the independent prognostic values of *KMT2C* and exon 9/20 *PIK3CA* pathogenic variants were comparable to that of established clinical and histopathological prognostic variables in patients with HPV-positive tumors (Figure 2 and Supplementary Figure 4). Older age (≥ 65 years) was significantly associated with longer DFS (HR = 0.52, 95%CI = [0.3, 0.92], p-value = 0.03) and MFS (HR = 0.52, 95%CI = [0.28, 0.96], p-value = 0.04). TNM staging variables and R1 resection margins were significantly associated with worse OS, DFS, and MFS. *PIK3CA* exon 9/20 variants were significantly associated with worse outcomes in all three measures of survival (OS: HR = 2.43, 95%CI = [1.3, 4.56], p-value = 0.006; DFS: HR = 1.81, 95%CI = [1.06, 3.08], p-value = 0.029; MFS: HR = 2.11, 95%CI = [1.19, 3.73], p-value = 0.010), as were *KMT2C* variants (OS: HR = 2.54, 95%CI = [1.25, 5.17], p-value = 0.010; DFS: HR = 3.38, 95%CI = [1.83, 6.26], p-value < 0.001; MFS: HR = 3.5, 95%CI = [1.85, 6.63], p-value < 0.001). In HPV-negative cases, no association between genomic alteration and survival outcomes reached statistical significance in multivariate analysis.

Actionability of alterations

Although most unique alterations were not targetable, a significant fraction of patients still had at least one mutation of theragnostic value in another cancer type found in their tumor. Of the 507 unique pathogenic variants analyzed for actionability, 9% (47/507), 2% (11/507), 1% (6/507), and 4% (21/507) were associated with Level 1, Level 2, Level 3B, and Level 4 evidence for therapeutic use, respectively (Figure 3A). Notably, targeted therapy backed with clinical evidence (*i.e.*, Level 1, Level 2, or Level 3B) could be matched to 43% (68/158) of patients in this cohort (Figure 3B). One or more alteration associated with FDA approval (i.e., Level 1 evidence) was detected in 35% of patients (55/158) with targetable *PIK3CA* alterations

(*i.e.*, p.(C420R), p.(E542K), p.(E545G), p.(E545K), or p.(H1047R)) being the most frequent (32/55, 58%). The second largest (20/55, 36%) was patients with alterations in Homologous Recombination Repair (HRR) genes (*i.e.*, *ATM*, *BARD1*, *BRCA1*, *BRCA2*, *BRIP1*, *CHEK2*, *FANCL*, *PALB2*, *RAD51C*, *RAD51D*). Other actionable alterations were found at low frequencies: *ERBB2* was altered by focal amplification in two tumors and affected by an activating mutation in another; *TSC1* and *TSC2* loss-of-function variants were found in two samples each. Of the patients who experienced relapse or died during the studied period, or experienced metastatic relapse or died during the study period, 44% (38/86) and 49% (37/76), respectively, carried variants that could be matched to targeted therapies with some clinical evidence for use in at least one cancer type.

Discussion

The genomic landscape of ASCC has been understudied and few in-depth molecular analyses have been published, depriving patients of the benefits of genomic medicine. This retrospective multicentric study offers a comprehensive tumor genomic profile of the largest cohort to date of patients diagnosed with ASCC who underwent APR after RT/CRT failure (*i.e.*, patients with the most severe clinical course). Analysis of variants of established pathogenicity identified recurrent alterations in genes and cellular pathways, with distinct patterns between HPV-positive and HPV-negative tumors. Frequent alterations were linked to survival outcomes, and the independent prognostic values of *KMT2C* and *PIK3CA* alterations in HPV-positive patients were comparable to that of established clinical and histopathological prognostic factors. Theragnostic annotation of pathogenic variants showed that 44% of patients with poor outcomes after APR could be matched to targeted therapies used in other cancers.

The genomic profiling of this cohort expands the findings of previous reports of ASCC molecular analyses, although methodological heterogeneities limit the possibility of direct

comparisons (18,21–23,35–38). For instance, Ito et al. (38) reported similar findings on a 30-patient subset of a Japanese cohort subjected to targeted tumor sequencing (*e.g.*, a high prevalence of the HPV-16 genotype and frequent *PIK3CA* exon 9 variants in HPV-positive cases). Intriguingly, they report 2 cases (6.7%) with *EGFR* activating mutations, absent in our cohort, with potential theragnostic implications. Conversely some key findings like *KMT2C* and *TERT* alterations observed in our study remain unaddressed in Ito's work due to the limited number of genes they tested.

On a broad scale, the most salient feature of the genomic landscape of ASCC is the differences between HPV-positive and HPV-negative tumors. In HPV-negative ASCC, a novel finding was the high prevalence of *TERT* promoter variants, which is consistent with what has been observed in other types of squamous cell carcinomas (39,40). This contrasts with the low frequency of *TERT* promoter variants in HPV-positive tumors, where telomerase activation is mediated by the E6 viral protein (41). Similarly, the high frequency of loss-of-function variants of *TP53* and *CDKN2A* in HPV-negative tumors parallels the inactivation of p53 and $p16^{INK4A}$-cyclin D1-RB pathways mediated by viral proteins E6 and E7 in HPV-positive tumors (42).

Chromatin remodeling genes were the most frequently altered genes in this cohort; chief among them were genes coding for proteins of the KMT2 family (*KMT2D*, *KMT2C*, and *KMT2B*), some of the most frequently mutated genes in cancer (43). Their primary biological function is regulating gene expression through methylation of histone H3K4 residues (44). Specifically, Kmt2d and Kmt2c regulate the activity of numerous enhancers (45), some of which control the expression of known tumor suppressors. They are also associated with ASCOM, a tumor-suppressive coactivator complex of p53 (46).

Pathogenic variants were found in ten of the 20 PI3K/AKT/mTOR pathway genes studied and in 61 tumors, all but one HPV-positive. These findings suggest that the previously reported

virtual absence of *PIK3CA* and *PTEN* alterations in HPV-negative tumors (18,21,35) could extend to the entire pathway. Interestingly, PI3K/AKT/mTOR pathway alterations were not significantly skewed towards HPV-positive tumors in head and neck squamous cell carcinomas (39,47–49). This suggest that HPV-negative ASCC carcinogenesis is either independent of PI3K/AKT/mTOR pathway activation or rely on non-genomic mechanisms (*e.g.*, epigenetic dysregulation) to achieve it. These findings show that while ASCC shares genomic features with squamous cell carcinomas, the interaction between HPV infection and genetic alterations may be unique and requires further study (24).

Our findings indicate that somatic *PIK3CA* exon 9/20 and *KMT2C* pathogenic variants are independent predictors of survival in patients with HPV-positive tumors. This is consistent with the study by Cacheux *et al.* (50), who also reported an association between *PIK3CA* alterations in exon 9/20 and shorter OS in patients treated by APR. However, other authors did not report a similar association between *PIK3CA* and survival outcomes (21,22,36,51). This could be attributed to previous studies describing heterogeneous cohorts of patients with discordant or unknown HPV status, or including non-exon 9/20 alterations in survival analyses. To our knowledge, this is the first time that *KMT2C* genomic alterations are identified as potential prognostic factor in ASCC patients, possibly because it is usually not included in smaller diagnostic NGS panels. Negative associations between alterations in this gene and survival have been suggested in non-small cell lung cancer (52), breast cancer (53), esophagogastric adenocarcinoma (54), and cutaneous squamous cell carcinoma (55). If validated in independent cohorts, *KMT2C* and *PIK3CA* status could be used to inform clinical prognostic assessments and risk stratification in future clinical trials for patients with ASCC treated with APR.

In addition to their potential prognostic power, the genomic alterations described here suggest multiple avenues of investigation of targeted therapies in ASCC. The current standard of care (concurrent 5-fluorouracil/mitomycin-based chemotherapy and radiotherapy) has been unchanged for decades (10,11). While immunotherapy is actively studied and shows promising results, the only therapeutic option offered to the approximately 35% of patients who experience locoregional relapse—the population described in this study—is APR, a mutilating surgery associated with high morbidity and mortality (7). In our cohort, 44% of patients had tumors with an alteration that could be matched to targeted therapies with some evidence for clinical use in other cancers. This suggests that—if those alterations were already detectable in early biopsies—almost half of the patients who experienced tumor persistence or recurrence after CRT could be candidates for clinical trials investigating genomic biomarker-driven treatments as a second line before APR. Furthermore, a potentially targetable variant was found in the tumor of 48% of patients who have already undergone APR and relapsed, opening the possibility of compassionate use for this group with few other therapeutic options.

The most frequent targetable alterations detected were activating mutation of *PIK3CA*, found in 25% (40/158) of tumors. Alpelisib, an α-specific PI3K inhibitor, has FDA approval for the treatment of hormone receptor-positive, HER2-negative, *PIK3CA* mutated advanced or metastatic breast cancer (56). At the time of the redaction of this manuscript, 12 ongoing clinical trials testing the safety or efficacy of alpelisib in squamous cell carcinoma are listed in the NIH clinical trial registry, yet none of them is open to ASCC patients (57). Another group of potentially targetable alterations is the inactivating variants of HRR genes, found in 13% (20/158) of patients in this cohort, including 6% (10/158) of *BRCA1* or *BRCA2* mutations. Those alterations sensitize cancer cells to poly ADP ribose polymerase inhibitors (PARPi) and are FDA-approved in BRCA mutated breast and ovarian cancers (58). The four PARPi currently used in the clinic (olaparib, rucaparib, talazoparib, and niraparib) are currently

investigated in at least one type of squamous cell carcinoma in 20 clinical trials. Still, only one (PEN-866-001) accepts ASCC patients. Interestingly, *in vitro* studies suggest that *KMT2C* and *KMT2D* pathogenic variants could sensitize tumor cells to PARPi (59,60). Another potential investigative avenue is the enzymatic inhibitor of EZH2 Tazemetostat, currently investigated in KTM2-mutated urothelial cancers (61). The potential therapeutic value of *PIK3CA* and *KMT2C* genomic alterations, combined with their potential prognostic power, makes them promising targets for further investigation in ASCC.

Limitations in this study must be considered when interpreting its results. The retrospective design and long inclusion period, due to the rarity of the disease, could introduce biases. The small number of HPV-negative cases limits statistical power and may have prevented the detection of rare genomic features. The highly selected nature of this cohort, consisting of the most severe ASCC patients, who underwent APR, could limit the generalizability of the results to early cases. Genomic analysis was conducted on FFPE samples without matched germline (as commonly done in clinical NGS testing), even though stringent filtering (see Methods section) and manual review ensured that virtually all alterations analyzed were bona fide somatic variants. Likewise, a stringent threshold on CNV detection was applied to offset the effect of FFPE-induced artifacts, reducing CNV detection sensitivity. Finally, only variants of known pathogenicity in genes with established roles in carcinogenesis have been studied, sacrificing discovery power for better biological and clinical interpretability.

Authors Contributions

CN, AL, and IB conceived the study and participated in its design. AL acquired the financial support for the project. MD, CN, JHL, MS, DG, PD, ES, MH, JL, J-FE, ER, VD-M, NR-L, CLF, DT, AC-G, PM, FP, WC, and AL provided patient samples and gathered and curated clinical data. IB and JM-P provided laboratory reagents and instrumentation. SV and CB

performed sequencing experiments. EG performed the bioinformatic analysis. AH analyzed the data, performed statistical analyses, created the visualizations, and wrote the original drafts. CN, AL, and IB reviewed the original drafts. The work reported in the paper has been performed by the authors, unless clearly specified in the text. All authors reviewed and approved the final manuscript.

Conflict of Interest

The authors declare no potential conflicts of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics statement

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and the General Data Protection Regulation (GDPR). The study was reviewed and approved by the Rennes University Hospital Ethics Committee for all participating centers (Registration No. 21.113). Following French regulations, formal informed consent was not required for this retrospective study; patients were informed of the study and did not express opposition.

Funding

This work was supported by the the Fondation de France and the Société Nationale Française de Gastroentérologie (Prix Pierre Durand et Marie Chevalier 2018) ; the Ligue Contre le Cancer (Appel d'Offre interrégional Grand Ouest 2019).

References

1. Nelson RA, Levine AM, Bernstein L, Smith DD, Lai LL. Changing Patterns of Anal Canal Carcinoma in the United States. JCO. 2013 Apr 20;31(12):1569–75.

2. Organisation mondiale de la santé, Centre international de recherche sur le cancer, editors. Digestive system tumours. 5th ed. Lyon: International agency for research on cancer; 2019. (World health organization classification of tumours).

3. Valmary-Degano S, Jacquin E, Prétet JL, Monnien F, Girardo B, Arbez-Gindre F, et al. Signature patterns of human papillomavirus type 16 in invasive anal carcinoma. Human Pathology. 2013 Jun;44(6):992–1002.

4. Kelly H, Chikandiwa A, Alemany Vilches L, Palefsky JM, de Sanjose S, Mayaud P. Association of antiretroviral therapy with anal high-risk human papillomavirus, anal intraepithelial neoplasia, and anal cancer in people living with HIV: a systematic review and meta-analysis. The Lancet HIV. 2020 Apr;7(4):e262–78.

5. Frisch M, Glimelius B, van den Brule AJC, Wohlfahrt J, Meijer CJLM, Walboomers JMM, et al. Sexually Transmitted Infection as a Cause of Anal Cancer. N Engl J Med. 1997 Nov 6;337(19):1350–8.

6. Daling JR, Madeleine MM, Johnson LG, Schwartz SM, Shera KA, Wurscher MA, et al. Human papillomavirus, smoking, and sexual practices in the etiology of anal cancer. Cancer. 2004 Jul 15;101(2):270–80.

7. Eng C, Messick C, Glynne-Jones R. The Management and Prevention of Anal Squamous Cell Carcinoma. American Society of Clinical Oncology Educational Book. 2019 May;(39):216–25.

8. Rao S, Guren MG, Khan K, Brown G, Renehan AG, Steigen SE, et al. Anal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Annals of Oncology. 2021 Sep;32(9):1087–100.

9. National Comprehensive Cancer Network. Anal Carcinoma (version 1.2022) [Internet]. [cited 2022 Jul 17]. Available from: https://www.nccn.org/professionals/physician_gls/pdf/anal.pdf

10. Bartelink H, Roelofsen F, Eschwege F, Rougier P, Bosset JF, Gonzalez DG, et al. Concomitant radiotherapy and chemotherapy is superior to radiotherapy alone in the treatment of locally advanced anal cancer: results of a phase III randomized trial of the European Organization for Research and Treatment of Cancer Radiotherapy and Gastrointestinal Cooperative Groups. JCO. 1997 May;15(5):2040–9.

11. Flam M, John M, Pajak TF, Petrelli N, Myerson R, Doggett S, et al. Role of mitomycin in combination with fluorouracil and radiotherapy, and of salvage chemoradiation in the definitive nonsurgical treatment of epidermoid carcinoma of the anal canal: results of a phase III randomized intergroup study. JCO. 1996 Sep;14(9):2527–39.

12. Schiller DE, Cummings BJ, Rai S, Le LW, Last L, Davey P, et al. Outcomes of Salvage Surgery for Squamous Cell Carcinoma of the Anal Canal. Ann Surg Oncol. 2007 Sep 19;14(10):2780–9.

13. Das P, Crane CH, Eng C, Ajani JA. Prognostic Factors for Squamous Cell Cancer of the Anal Canal. 2008;2(1):5.

14. Bruyere D, Monnien F, Colpart P, Roncarati P, Vuitton L, Hendrick E, et al. Treatment algorithm and prognostic factors for patients with stage I–III carcinoma of the anal canal: a 20-year multicenter study. Mod Pathol. 2021 Jan;34(1):116–30.

15. Meulendijks D, Tomasoa NB, Dewit L, Smits PHM, Bakker R, van Velthuysen MLF, et al. HPV-negative squamous cell carcinoma of the anal canal is unresponsive to standard treatment and frequently carries disruptive mutations in TP53. Br J Cancer. 2015 Apr;112(8):1358–66.

16. Parwaiz I, MacCabe TA, Thomas MG, Messenger DE. A Systematic Review and Meta-Analysis of Prognostic Biomarkers in Anal Squamous Cell Carcinoma Treated With Primary Chemoradiotherapy. Clinical Oncology. 2019 Dec;31(12):e1–13.

17. National Cancer Institute. The Cancer Genome Atlas Program [Internet]. Available from: https://www.cancer.gov/tcga

18. Aldersley J, Lorenz DR, Mouw KW, D'Andrea AD, Gabuzda D. Genomic Landscape of Primary and Recurrent Anal Squamous Cell Carcinomas in Relation to HPV Integration, Copy-Number Variation, and DNA Damage Response Genes. Molecular Cancer Research. 2021 Aug 1;19(8):1308–21.

19. Cacheux W, Dangles-Marie V, Rouleau E, Lazartigues J, Girard E, Briaux A, et al. Exome sequencing reveals aberrant signalling pathways as hallmark of treatment-naive anal squamous cell carcinoma. Oncotarget. 2018 Jan 2;9(1):464–76.

20. Chung JH, Sanford E, Johnson A, Klempner SJ, Schrock AB, Palma NA, et al. Comprehensive genomic profiling of anal squamous cell carcinoma reveals distinct genomically defined classes. Annals of Oncology. 2016 Jul;27(7):1336–41.

21. Morris V, Rao X, Pickering C, Foo WC, Rashid A, Eterovic K, et al. Comprehensive Genomic Profiling of Metastatic Squamous Cell Carcinoma of the Anal Canal. Mol Cancer Res. 2017 Nov;15(11):1542–50.

22. Trilla-Fuertes L, Ghanem I, Maurel J, G-Pastrián L, Mendiola M, Peña C, et al. Comprehensive Characterization of the Mutational Landscape in Localized Anal Squamous Cell Carcinoma. Translational Oncology. 2020 Jul;13(7):100778.

23. Zhu X, Jamshed S, Zou J, Azar A, Meng X, Bathini V, et al. Molecular and immunophenotypic characterization of anal squamous cell carcinoma reveals distinct clinicopathologic groups associated with HPV and TP53 mutation status. Mod Pathol. 2021 May;34(5):1017–30.

24. Sánchez-Danés A, Blanpain C. Deciphering the cells of origin of squamous cell carcinomas. Nat Rev Cancer. 2018 Sep;18(9):549–61.

25. Moreira A, Poulet A, Masliah-Planchon J, Lecerf C, Vacher S, Larbi Chérif L, et al. Prognostic value of tumor mutational burden in patients with oral cavity squamous cell carcinoma treated with upfront surgery. ESMO Open. 2021 Aug;6(4):100178.

26. Genome Aggregation Database Consortium, Karczewski KJ, Francioli LC, Tiao G, Cummings BB, Alföldi J, et al. The mutational constraint spectrum quantified from variation in 141,456 humans. Nature. 2020 May 28;581(7809):434–43.

27. Niu B, Ye K, Zhang Q, Lu C, Xie M, McLellan MD, et al. MSIsensor: microsatellite instability detection using paired tumor-normal sequence data. Bioinformatics. 2014 Apr 1;30(7):1015–6.

28. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, et al. Integrative Analysis of Complex Cancer Genomics and Clinical Profiles Using the cBioPortal. Science Signaling. 2013 Apr 2;6(269):pl1–pl1.

29. Chakravarty D, Gao J, Phillips S, Kundra R, Zhang H, Wang J, et al. OncoKB: A Precision Oncology Knowledge Base. JCO Precision Oncology. 2017 Nov;(1):1–16.

30. Sanchez-Vega F, Mina M, Armenia J, Chatila WK, Luna A, La KC, et al. Oncogenic Signaling Pathways in The Cancer Genome Atlas. Cell. 2018 Apr;173(2):321-337.e10.

31. Tate JG, Bamford S, Jubb HC, Sondka Z, Beare DM, Bindal N, et al. COSMIC: the Catalogue Of Somatic Mutations In Cancer. Nucleic Acids Research. 2019 Jan 8;47(D1):D941–7.

32. oncokb-annotator [Internet]. Available from: https://github.com/oncokb/oncokb-annotator#readme

33. Canisius S, Martens JWM, Wessels LFA. A novel independence test for somatic alterations in cancer shows that biology drives mutual exclusivity but chance explains most co-occurrence. Genome Biol. 2016 Dec;17(1):261.

34. Bellera CA, Pulido M, Gourgou S, Collette L, Doussau A, Kramar A, et al. Protocol of the Definition for the Assessment of Time-to-event Endpoints in CANcer trials (DATECAN) project: Formal consensus method for the development of guidelines for standardised time-to-event endpoints' definitions in cancer clinical trials. European Journal of Cancer. 2013 Mar;49(4):769–81.

35. Iseas S, Golubicki M, Robbio J, Ruiz G, Guerra F, Mariani J, et al. A clinical and molecular portrait of non-metastatic anal squamous cell carcinoma. Translational Oncology. 2021 Jun;14(6):101084.

36. Moniz CMV, Riechelmann RP, Oliveira SCR, Bariani GM, Rivelli TG, Ortega C, et al. A Prospective Cohort Study of Biomarkers in Squamous Cell Carcinoma of the Anal Canal (SCCAC) and their Influence on Treatment Outcomes. J Cancer. 2021;12(23):7018–25.

37. Smaglo BG, Tesfaye A, Halfdanarson TR, Wang J, Gatalica Z, Reddy S, et al. Comprehensive multiplatform biomarker analysis of 199 anal squamous cell carcinomas. :11.

38. Ito T, Takayanagi D, Sekine S, Hashimoto T, Shimada Y, Matsuda M, et al. Comparison of clinicopathological and genomic profiles in anal squamous cell carcinoma between Japanese and Caucasian cohorts. Sci Rep. 2023 Mar 3;13(1):3587.

39. Morris LGT, Chandramohan R, West L, Zehir A, Chakravarty D, Pfister DG, et al. The Molecular Landscape of Recurrent and Metastatic Head and Neck Cancers: Insights From a Precision Oncology Sequencing Platform. JAMA Oncol. 2017 Feb 1;3(2):244.

40. PCAWG Pathogens, PCAWG Consortium, Zapatka M, Borozan I, Brewer DS, Iskar M, et al. The landscape of viral associations in human cancers. Nat Genet. 2020 Mar;52(3):320–30.

41. Liu X, Dakic A, Zhang Y, Dai Y, Chen R, Schlegel R. HPV E6 protein interacts physically and functionally with the cellular telomerase complex. Proceedings of the National Academy of Sciences. 2009 Nov 3;106(44):18780–5.

42. Smeets SJ, van der Plas M, Schaaij-Visser TBM, van Veen EAM, van Meerloo J, Braakhuis BJM, et al. Immortalization of oral keratinocytes by functional inactivation of the p53 and pRb pathways. Int J Cancer. 2011 Apr 1;128(7):1596–605.

43. Kandoth C, McLellan MD, Vandin F, Ye K, Niu B, Lu C, et al. Mutational landscape and significance across 12 major cancer types. Nature. 2013 Oct 17;502(7471):333–9.

44. Rao RC, Dou Y. Hijacked in cancer: the KMT2 (MLL) family of methyltransferases. Nat Rev Cancer. 2015 Jun;15(6):334–46.

45. Hu D, Gao X, Morgan MA, Herz HM, Smith ER, Shilatifard A. The MLL3/MLL4 Branches of the COMPASS Family Function as Major Histone H3K4 Monomethylases at Enhancers. Mol Cell Biol. 2013 Dec;33(23):4745–54.

46. Lee J, Kim DH, Lee S, Yang QH, Lee DK, Lee SK, et al. A tumor suppressive coactivator complex of p53 containing ASC-2 and histone H3-lysine-4 methyltransferase MLL3 or its paralogue MLL4. Proceedings of the National Academy of Sciences. 2009 May 26;106(21):8513–8.

47. Dubot C, Bernard V, Sablin MP, Vacher S, Chemlali W, Schnitzler A, et al. Comprehensive genomic profiling of head and neck squamous cell carcinoma reveals FGFR1 amplifications and tumour genomic alterations burden as prognostic biomarkers of survival. European Journal of Cancer. 2018 Mar;91:47–55.

48. Lechner M, Frampton GM, Fenton T, Feber A, Palmer G, Jay A, et al. Targeted next-generation sequencing of head and neck squamous cell carcinoma identifies novel genetic alterations in HPV+ and HPV- tumors. Genome Med. 2013;5(5):49.

49. The Cancer Genome Atlas Network. Comprehensive genomic characterization of head and neck squamous cell carcinomas. Nature. 2015 Jan 29;517(7536):576–82.

50. Cacheux W, Rouleau E, Briaux A, Tsantoulis P, Mariani P, Richard-Molard M, et al. Mutational analysis of anal cancers demonstrates frequent PIK3CA mutations associated with poor outcome after salvage abdominoperineal resection. Br J Cancer. 2016 Jun;114(12):1387–94.

51. Casadei Gardini A, Capelli L, Ulivi P, Giannini M, Freier E, Tamberi S, et al. KRAS, BRAF and PIK3CA Status in Squamous Cell Anal Carcinoma (SCAC). de Mello RA, editor. PLoS ONE. 2014 Mar 18;9(3):e92071.

52. Luo LY, Samstein RM, Dick-Godfrey R, Sidiqi B, Wang C, Oro F, et al. Genomic Analyses for Predictors of Response to Chemoradiation in Stage III Non-Small Cell Lung Cancer. Advances in Radiation Oncology. 2021 Jan;6(1):100615.

53. Liu X, Qiu R, Xu M, Meng M, Zhao S, Ji J, et al. KMT2C is a potential biomarker of prognosis and chemotherapy sensitivity in breast cancer. Breast Cancer Res Treat. 2021 Sep;189(2):347–61.

54. Hao D, He S, Harada K, Pizzi MP, Lu Y, Guan P, et al. Integrated genomic profiling and modelling for risk stratification in patients with advanced oesophagogastric adenocarcinoma. Gut. 2021 Nov;70(11):2055–65.

55. Pickering CR, Zhou JH, Lee JJ, Drummond JA, Peng SA, Saade RE, et al. Mutational Landscape of Aggressive Cutaneous Squamous Cell Carcinoma. Clin Cancer Res. 2014 Dec 15;20(24):6582–92.

56. Narayan P, Prowell TM, Gao JJ, Fernandes LL, Li E, Jiang X, et al. FDA Approval Summary: Alpelisib Plus Fulvestrant for Patients with HR-positive, HER2-negative, PIK3CA-mutated, Advanced or Metastatic Breast Cancer. Clinical Cancer Research. 2021 Apr 1;27(7):1842–9.

57. National Institute of Health. ClinicalTrials.gov [Internet]. [cited 2021 Sep 18]. Available from: https://clinicaltrials.gov/ct2/results?cond=Squamous+Cell+Carcinoma&term=alpelisib&cntry=&state=&city=&dist=

58. Lord CJ, Ashworth A. PARP inhibitors: Synthetic lethality in the clinic. Science. 2017 Mar 17;355(6330):1152–8.

59. Chang A, Liu L, Ashby JM, Wu D, Chen Y, O'Neill SS, et al. Recruitment of KMT2C/MLL3 to DNA Damage Sites Mediates DNA Damage Responses and Regulates PARP Inhibitor Sensitivity in Cancer. Cancer Res. 2021 Jun 15;81(12):3358–73.

60. Rampias T, Karagiannis D, Avgeris M, Polyzos A, Kokkalis A, Kanaki Z, et al. The lysine-specific methyltransferase KMT 2C/ MLL 3 regulates DNA repair components in cancer. EMBO Rep [Internet]. 2019 Mar [cited 2022 Feb 20];20(3). Available from: https://onlinelibrary.wiley.com/doi/10.15252/embr.201846821

61. Meeks JJ, Shilatifard A, Miller SD, Morgans AK, VanderWeele DJ, Kocherginsky M, et al. A pilot study of tazemetostat and MK-3475 (pembrolizumab) in advanced urothelial

carcinoma (ETCTN 10183). Journal of Clinical Oncology. 2020;38(6_suppl):TPS607–TPS607.

Tables and figures

Table captions

Table 1: Clinical and histopathological characteristics of the cohort.

HIV: human immunodeficiency virus, HPV: Human papillomavirus.

Figures captions

Figure 1: Genomic landscape of ASCC.

Samples are divided by HPV status. Truncating variants include non-sense, frame-shift variants, and substitutions at splice-sites in tumor suppressor genes. Only variants with established pathogenicity (determined as described in the Material and Methods section) are represented.

Figure 2: *KMT2C* and *PIK3CA* exon 9/20 alterations are associated with shorter OS in patients with HPV-positive tumors.

(A) Cox multivariate model of OS; (B) Kaplan-Meier curves of OS stratified by mutational status. The p-value of the log-rank test comparing survival probabilities is represented on the graph. WT: wild type.

Figure 3: Theragnostic annotation of genomic alterations reveals that ASCC is a target-rich malignancy.

(A) Highest level of actionability in another cancer of the 507 unique pathogenic variants found in this cohort as determined by the OncoKB classification system (29); (B) Highest level of actionability associated with variants found in each patient's tumor. Level 1: Biomarker recognized by the FDA to be predictive of a response to an FDA-approved drug; Level 2: Biomarker identified as a standard of care biomarker by the NCCN or other professional

guidelines to be predictive of response to an FDA-approved drug; Level 3B: Compelling clinical evidence, as determined by OncoKB curators, supporting the theragnostic value of the biomarker in any other cancer; Level 4: Compelling biological evidence, as determined by OncoKB curators, supporting the theragnostic value of the biomarker.

Table 1: Clinical and histopathological characteristics of the cohort.

Characteristic	N	Overall, N = 158	HPV status		p-value[2]
			Positive, N = 141[1]	Negative, N = 17[1]	
Age	158	57 (49, 66)[1]	58 (50, 66)	52 (49, 62)	0.43
Sexe	158				**0.001**
Female		102 (65%)	97 (69%)	5 (29%)	
Male		56 (35%)	44 (31%)	12 (71%)	
Stage	145				0.47
I		14 (10%)	11 (9%)	3 (20%)	
II		70 (48%)	63 (48%)	7 (47%)	
III		59 (41%)	54 (42%)	5 (33%)	
IV		2 (1%)	2 (2%)	0 (0%)	
ypT	156				0.15
ypT1		19 (12%)	19 (14%)	0 (0%)	
ypT2		60 (38%)	50 (36%)	10 (59%)	
ypT3		32 (21%)	28 (20%)	4 (24%)	
ypT4		45 (29%)	42 (30%)	3 (18%)	
ypN	156				0.2
ypN0		125 (80%)	110 (79%)	15 (94%)	
ypN+		31 (20%)	30 (21%)	1 (6%)	
Differentiation	156				0.36
Low		36 (23%)	34 (24%)	2 (12%)	
Moderate/high		120 (77%)	105 (76%)	15 (88%)	
Vascular embols	157	58 (37%)	54 (39%)	4 (24%)	0.22
Resection margin	157				0.2
R0		123 (78%)	108 (77%)	15 (94%)	
R1		34 (22%)	33 (23%)	1 (6%)	
Pre-operative treatment	156				**0.018**
Chemoradiotherapy		111 (71%)	104 (74%)	7 (44%)	
Radiotherapy		45 (29%)	36 (26%)	9 (56%)	
Idication for Surgery	158				**0.004**
Tumor persistence		43 (27%)	33 (23%)	10 (59%)	
Tumor reccurence		115 (73%)	108 (77%)	7 (41%)	
HIV status	143				>0.99
Negative		120 (84%)	105 (83%)	15 (88%)	
Positive		23 (16%)	21 (17%)	2 (12%)	
HPV serotype	158				
Negative		17 (11%)		17 (100%)	
Serotype 16		126 (80%)	126 (89%)		
Serotype 18		5 (3%)	5 (3%)		
Other serotype		10 (6%)	10 (7%)		

[1]: Median (IQR) for continuous variables; n (%) for categorical variables

[2]: Wilcoxon rank sum test for continuous variables, Fisher's exact test for categorical variables

N: number of patient with non missing data for the variable; HIV: human immunodeficiency virus; HPV: Human papillomavirus.

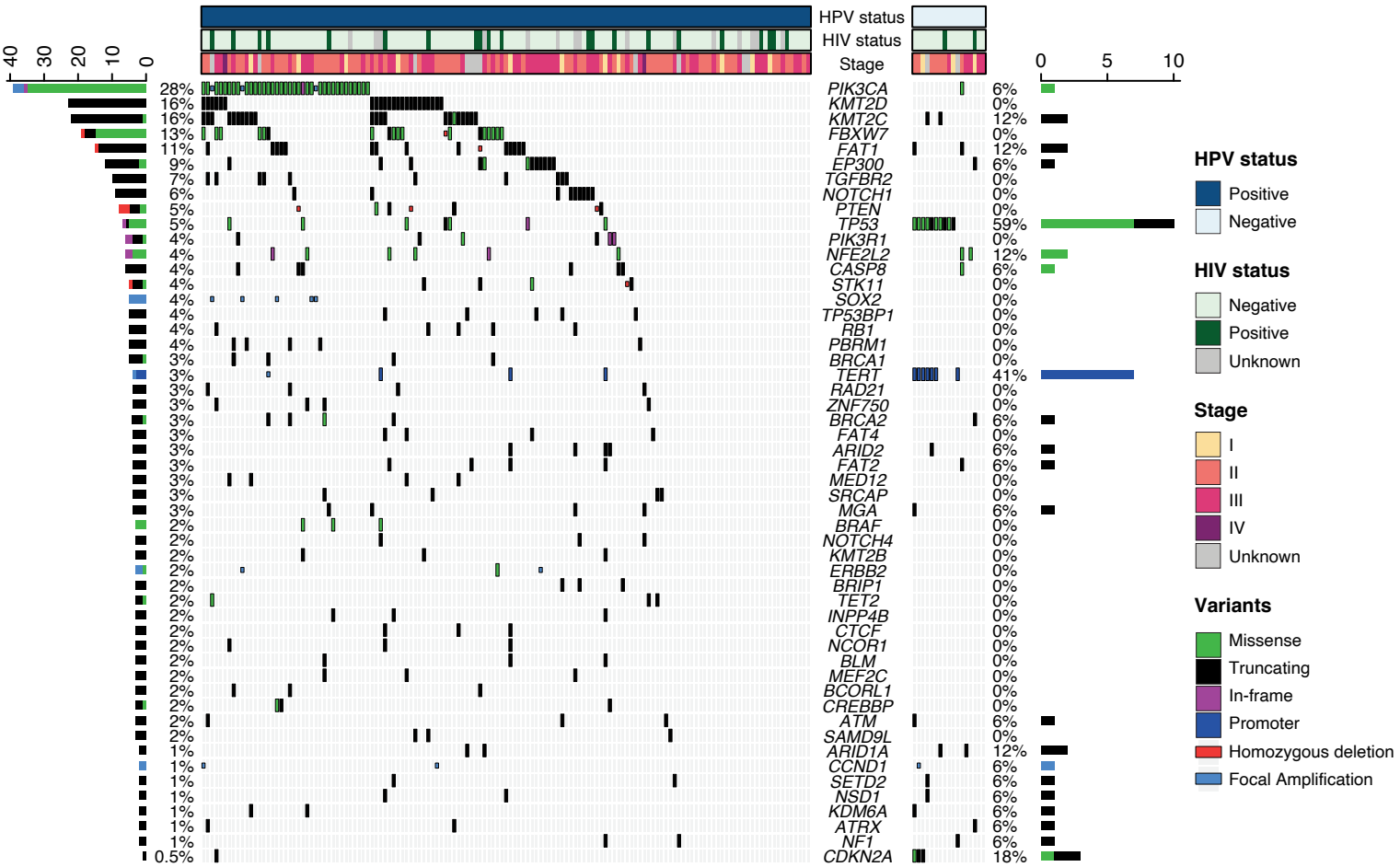
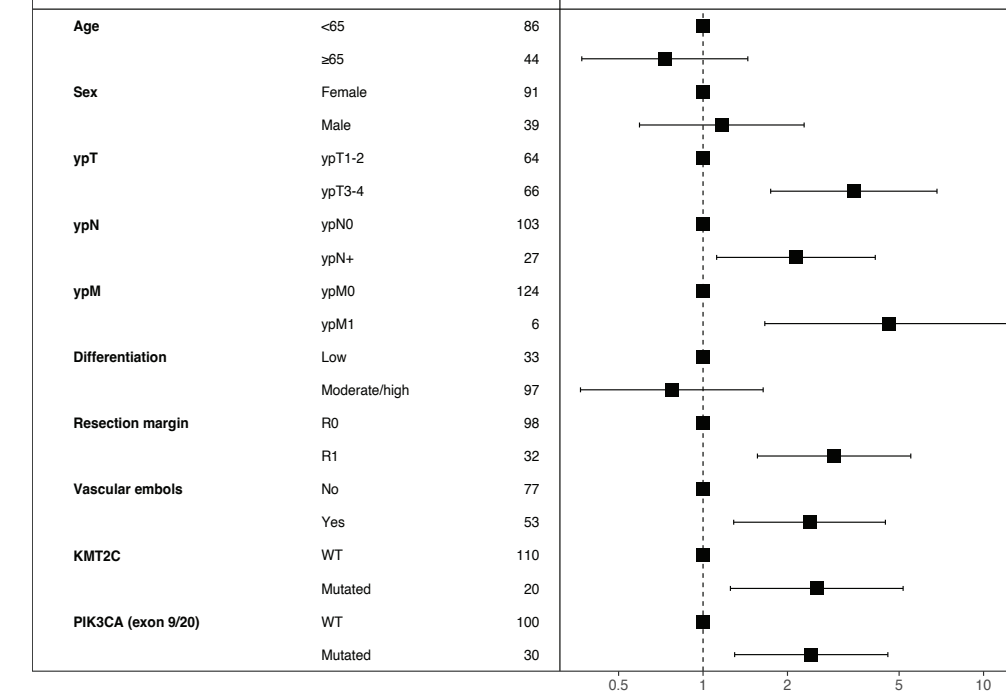


Figure 1
Hamza et al.

(A)

Variable		N	Hazard ratio	p-value
Age	<65	86	Reference	
	≥65	44	0.73 (0.37, 1.44)	0.367
Sex	Female	91	Reference	
	Male	39	1.17 (0.59, 2.29)	0.655
ypT	ypT1-2	64	Reference	
	ypT3-4	66	3.45 (1.74, 6.82)	<0.001
ypN	ypN0	103	Reference	
	ypN+	27	2.14 (1.12, 4.11)	0.022
ypM	ypM0	124	Reference	
	ypM1	6	4.60 (1.66, 12.73)	0.003
Differentiation	Low	33	Reference	
	Moderate/high	97	0.77 (0.37, 1.64)	0.503
Resection margin	R0	98	Reference	
	R1	32	2.93 (1.56, 5.50)	<0.001
Vascular embols	No	77	Reference	
	Yes	53	2.40 (1.29, 4.47)	0.006
KMT2C	WT	110	Reference	
	Mutated	20	2.54 (1.25, 5.17)	0.010
PIK3CA (exon 9/20)	WT	100	Reference	
	Mutated	30	2.43 (1.30, 4.56)	0.006



(B)

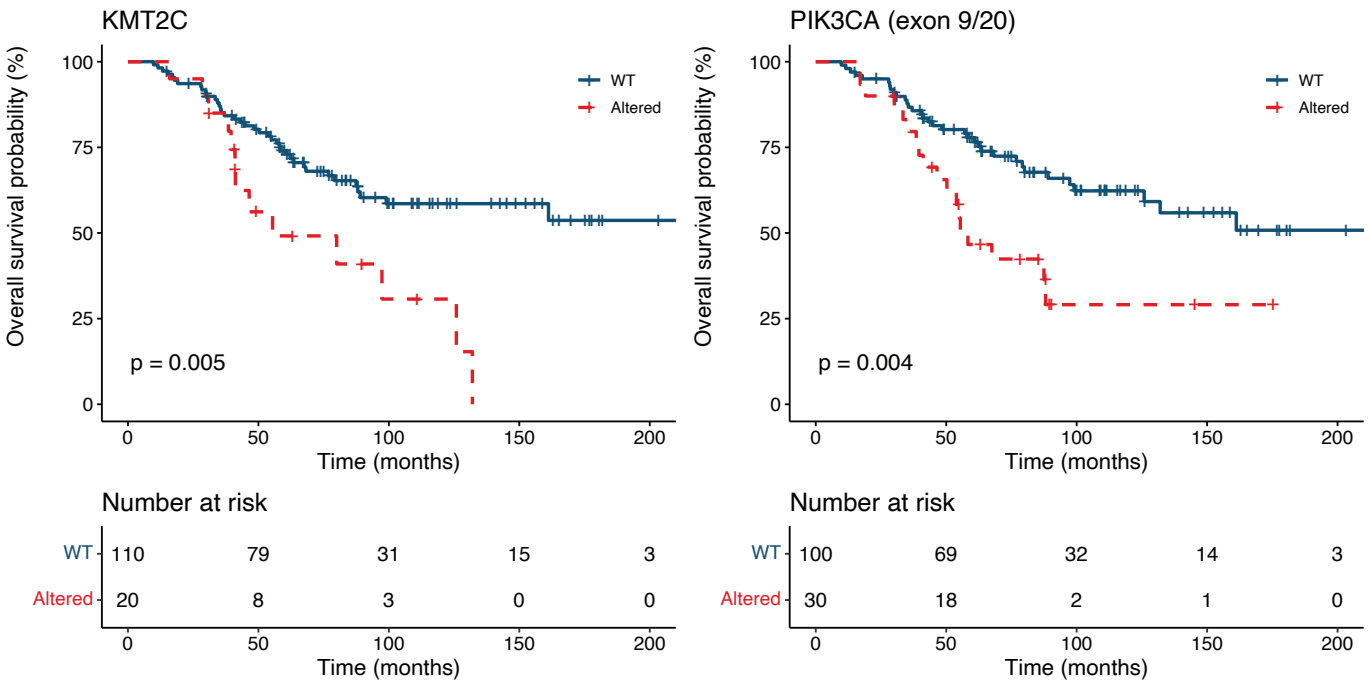


Figure 2

Hamza *et al.*

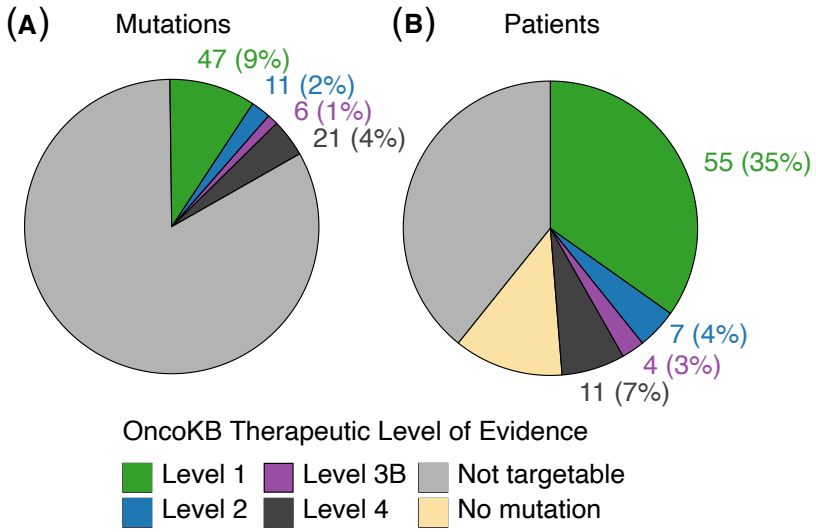


Figure 3
Hamza et al