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Sex-Based Difference in Gene Alterations and Biomarkers in Anal Squamous Cell Carcinoma

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Highlights

- To our knowledge, this is the largest genetic profiling study in ASCC to date.
- We observed differences in the prevalence of HPV-genotype based on sex.
- Genomic profiling of ASCC examined in this study showed different alterations between men and women.
- Some of the alterations identified (e.g. PIK3CA) potentially make this disease eligible to target therapy.
- The high prevalence of a favorable immunologic profile could be predictive of response to immunotherapy.

1. Abstract

1.1. Background: anal squamous cell carcinoma (ASCC) is a relatively rare malignancy ac-counting for about 2-3% of all the gastrointestinal tumors. The standard of treatment for localized disease is chemoradiotherapy. Several studies reported a sex disparity in ASCC prognosis showing a better survival for female compared to men. Methods: we examined 1,380 patients with ASCC who received comprehensive genomic profiling as part of routine clinical care and present key differences in mutational patterns based on sex and human papilloma virus status. Results: HPV-16 is identified more frequently in female patients, whereas HPV-6 incidence was higher in male patients. Male patients had higher rates of alterations for TERT, TP53, and CDKN2A, while alterations in CBFB were nearly exclusively found in female HPV(+) patients. Approximately 30% of patients have alteration of PIK3CA. Over 70% of the population was positive for PD-L1 (1%+), with nearly 20% exhibiting a high PDL-1 expression (50%+). Additionally, ~18% of patients had a high tumor mutational burden (TMB>=10 mutations/Mb). Conclusions: high rates of PI3K gene alteration and favorable immunological profiles observed in our study suggest possible thera-peutic opportunities in which ASCC patients may benefit from newfound precision treatments and immunotherapy in addition to, or in lieu of, standard-of-care chemoradiotherapy.

2. Introduction

Anal cancer is an uncommon disease representing about 2-3% of all the gastrointestinal cancer. However, its incidence has been increasing over time in the US and developed countries [1-4]. In fact, although still rare in the general population, a high incidence has been noted in specific population groups due to a change of sexual norms and behavior, as well as the emergence of HIV and the increases in female smoking. Conversely, with the emergence of the vaccines for papillomavirus, the younger adult generation now would be less predisposed ASCC. The prevalence is higher in patients with HIV, men who have sex with men, recipients of solid organ transplants, women with genital neoplasia, and patients with systemic lupus erythematosus or inflammatory bowel dis-

ease. The higher incidence among individuals who are HIV-positive makes anal squamous cell carcinoma one of the most common non-AIDS-defining cancers among HIV-positive individuals [1]. Anal cancer often presents with bleeding; however, diagnosis may be delayed because bleeding is attributed to hemorrhoids. It may also present with any combination of a mass, non-healing ulcer, pain, bleeding, itching, discharge, faecal incontinence, and fistulae. Digital anorectal examination is an important clinical tool for detection of lesions in the anal area. The diagnosis of anal cancer is made by biopsy-proven histology [1].

Anal squamous carcinoma (ASCC) is the most prevalent histological subtype, representing about 80% of this cancer [1]. The standard of treatment for localized disease is chemotherapy with 5-fluorouracil and mitomycin-C in association with radiotherapy (total dose 50.4 to 54 Gy) [5]. Chemotherapy with platinum and fluorouracil is the standard of care in metastatic setting, which accounts for less than 10% of ASCC at diagnosis [1]. Human papilloma virus (HPV) infection is the most important recognized risk factor associated with ASCC, especially HPV-16 and HPV-18 subtypes. Age, disease stage, smoking, location, immunosuppressive drugs, and p16 status are other known prognostic factors [6].

Several studies have reported a sex-based disparity in ASCC prognosis [1,5,6], several of which have shown better overall survival (OS) in females compared to males: a survival advantage of about 15% (69% vs 55%) [7]. No mechanism has been definitively shown to explain this [8-10]. We sought to investigate a large real-world cohort comprising 1,380 patients with ASCC in order to identify and quantify sex-based differences in cancer-associated gene alterations that may better inform future precision medicine initiatives and provide insights on the potential differences in survival based on sex in ASCC.

3. Materials and Methods

3.1. Comprehensive Genomic Profiling of ASCC

This study comprised a cohort of 1,380 ASCC cases. Formalin-fixed, paraffin embedded (FFPE) tissue biopsies were submitted for comprehensive genomic profiling (CGP) us-ing FoundationOne® or FoundationOne®CDx. All samples were assessed in Clinical Laboratory Improvement Amendments (CLIA)-certified and College of American Pathologists (CAP)-accredited laboratories. Using a minimum of 50 ng of DNA, CGP was performed on hybridization-captured, adaptor ligation-based libraries ran on Il-lumina HiSeq 4000 Systems to a median exon coverage depth of > 500X for select exons and introns of at least 324 cancer-related genes. The analysis was limited to the 296 genes commonly targeted in the two assays evaluated for this study. The detection of genomic alterations, including short variants (base substitutions, insertions, and dele-tions), copy number alterations (focal amplifications and homozygous deletions), and select gene fusions or rearrangements was performed as described previously [11,12]. Tumor mutational burden (TMB, mutations/Mb) was calculated based on sequences of 0.8–1.1 Mb of DNA [13] and microsatellite instability was determined by analyzing in-tronic homopolymer repeat loci for length variability and compiled into an overall MSI score [14]. Patient age, sex, and site of biopsy were extracted from accompanying pa-thology reports or submitted test requisition form. PD-L1 expression was determined by IHC performed on FFPE tissue sections using the Dako 22C3 PD-L1 antibody (tumor proportion score, TPS), according to the manufacturer's instructions. Approval for this study, including a waiver of informed consent and Health Insurance Portability and Ac-countability Act waiver of authorization, was obtained from the Western Institutional Review Board (Protocol #20152817).

3.2. Viral Detection

A computational algorithm leveraging the sequencing coverage on a list of pathogens, designed in the hybrid capture assay, was used (PMID: 23718828). Cases with HPV-16, HPV-18, HPV-6, or HPV-11 detected were denoted as HPV(+) in this study. Differences in the genomic landscape of HPV(+) and HPV(-) ASCC were examined based on sex.

3.3. Statistical Procedures

Lower and upper 95% confidence intervals for prevalence of gene alterations and other biomarkers were calculated using a percent point function (scipy). Differences in the prevalence of gene alterations and biomarkers were assessed using a Fisher's exact test with false discovery rate (FDR)-based correction for multiple testing. Significance has been set at $p \leq 0.05$.

4. Results

This study investigated 1,380 ASCC cases (Table 1), comprising 952 female and 428 male patients. Median age was 61 years; 582 (42.4%) were biopsied from a local site (anus, rectum, colon), 499 (36.4%) were obtained from metastatic biopsies, 176 (12.8%) were biopsied from a lymph node, while limited information was available for the remaining 115 (8.4%). 582 (42.4%) had a local disease; 499 (36.2%) metastatic disease and 176 (12.8%) lymph node metastasis alone. No difference in age or biopsy site at the time of diagnosis was observed according to sex and HPV status (Table 1). HPV positive status was more frequently present in females compared to males (89.3% vs 73.6%; p <10-3). HPV-16 is the most expressed HPV genotype (78.3% of cases overall) with a higher rate in females (84.2% vs 65.2%; p < 10-3). HPV-18 was identified to be similarly prevalent in the male and female patients (4.2% in each) (p = 1.0). Though relatively rarer, HPV-6(+) incidence was significantly male-skewed (16/428 male patients, 3.7% vs. 7/952 female patients, 0.7%; p < 10-3).

Next, we examined the immunological profile of ASCC based on sex and HPV status (Table 2). PD-L1 was highly expressed in the entire population, with over 70% of cases showing at least 1% IHC staining (Table 2). A slightly higher rate of PD-L1 positivity

was observed in HPV positive cases compared to HPV negative cases, although it did not reach statistical significance (74.5% vs. 63.9%; p = 0.16). Similar rates were observed in female and male patients (74.0% vs. 70.4%; p = 0.80) (Table 2). A high positive PD-L1 stain, defined at ≥ 50%, was observed in about 20% of the population with neither sex (p = 0.80) nor HPV expression (p = 0.34) showing notable differences. Of note, in HPV negative tumors, PD-L1 ≥ 50% was seen in 13.3% of females compared to 31.0% of males, albeit limited by cohort size (p = 0.83). Concurrently, high tumor mutational burden (TMB-High, ≥ 10 mutations/Mb) was found in about 18% of the population without significant differences according to sex (p = 0.80). A slightly elevated rate of TMB-high was observed in HPV positive cases compared to HPV negative cases (18.4% vs. 14.0%; p = 0.16), albeit limited by cohort size. In general, high microsatellite instability (MSI-H) was rare, present in less than 1% of the entire population (Table 2).

We next interrogated differences in gene alterations between the different subgroups of ASCC based on sex and HPV status (Table 3). PIK3CA alterations were detected in 34% of patients with a higher prevalence in the HPV positive population compared to the HPV negative population (37.1% vs. 16.8%; p < 10-3), with similar differences seen in each sex (Females: 37.1% vs. 20.8%; Males: 37.1% vs. 13.3%). In contrast, some gene alterations were less prevalent in the HPV positive population compared to the HPV negative population: TP53 (5.6% vs 44.9%, p < 10-3), TERT (2.7% vs 31.3%, p < 10-3), CDNK2A (2.9% vs 29.0%, p < 10-3), CDNK2B (1.6% vs 11.7%, p < 10-3) and NOTCH1 (5.1% vs 14.5%, p < 10-3). Based on sex, the female population presented with higher rates of alterations in PTEN (15.3% vs 8.4%; p=0.003), KMT2D (21.0% vs 13.1%, p=0.003) and CBFB (3.3% vs 0.2%; p=0.002); CREBBP was also seen more frequently in female patients compared to male patients, however it did not reach statistical significance after FDR correction (5.4 vs 2.6%; p = 0.062) (Table 3). In contrast, many gene alterations were more prevalent in male patients including TERT (15.2% vs 3.5%, p < 10-3), TP53 (20.3% vs 7.8%, p < 10-3), CDKN2A (14.0% vs 3.8%, p <10-3) , CDKN2B (5.4% vs 2.2%; p = 0.021), MYC (4.2% vs 1.6%; p=0.027) as well as amplifications on 11q13 such as CCND1 (7.0% vs 3.1%, p = 0.017), FGF19 (7.0% vs 3.3%, p = 0.017), FGF3 (7.2% vs 3.6%, p = 0.021), FGF4 (7.0% vs 3.5%, p = 0.023). Of note, BRCA1 and BRCA2 mutations were present in approximately 5% of the population with a mild male predominance (~7% vs ~4%) regardless of HPV expression, although it did not reach statistical significance. Further, even within the HPV negative population, we observed sex-based differences. Alterations in TP53, TERT and CDKN2A, continued to be more prevalent in male patients compared to female patients within the HPV negative population. However, we did not observe any differences between male and female subpopulations in the HPV-positive cohort, with the exception of CBFB. Overall, the findings presented in our study outline key sex and HPV-status associated genomic and immunogenic profiles in ASCC (Table 3).

Table 1: Patient cohort characteristics according to sex and HPV status among 1,380 cases with ASCC

	OVERALL							HPV(+)			HPV(-)		
Cohort	**ASCC (all) (N=1,380)**	**Female (N=952)**	**Male (N=428)**	**P value (Female vs. Male)**	**HPV(+) (N=1,166)**	**HPV(-) (N=214)**	**P value (HPV(+) vs. HPV(-))**	**Female HPV(+) (N=851)**	**Male HPV(+) (N=315)**	**P value (Female vs. Male)**	**Female HPV(-) (N=101)**	**Male HPV(-) (N=113)**	**P value (Female vs. Male)**
Age, median (IQR)	61 (54-68)	61 (55-68)	60 (54-68)		61 (54-68)	63 (56-70.75)		61 (54-68)	60 (53-67)		63 (58-70)	63 (55-71)	
Evaluable for Biopsy Site	1372	945	427		1161	211		846	315		99	112	
Local	582 (42.2%)	397 (41.7%)	185 (43.2%)	0.75	484 (41.5%)	98 (45.8%)	0.32	348 (40.9%)	136 (43.2%)	0.63	49 (48.5%)	49 (43.4%)	1
Metastatic (non-Lymph Node)	499 (36.2%)	348 (36.6%)	151 (35.3%)	0.75	438 (37.6%)	61 (28.5%)	2.60E-02	320 (37.6%)	118 (37.5%)	1	28 (27.7%)	33 (29.2%)	1
Lymph Node	176 (12.8%)	117 (12.3%)	59 (13.8%)	0.66	149 (12.8%)	27 (12.6%)	1	107 (12.6%)	42 (13.3%)	0.85	10 (9.9%)	17 (15.0%)	1
Unknown/ Uncertain	115 (8.3%)	83 (8.7%)	32 (7.5%)	0.66	90 (7.7%)	25 (11.7%)	0.085	71 (8.3%)	19 (6.0%)	0.36	12 (11.9%)	13 (11.5%)	1
HPV 16/18(+) (high risk strains)	1,139 (82.5%)	842 (88.4%)	297 (69.4%)	1.00E-15	1,139 (97.7%)	0 (0.00%)	3.90E-221	842 (98.9%)	297 (94.3%)	7.00E-05	0 (0.00%)	0 (0.00%)	1
HPV-16	1,081 (78.3%)	802 (84.2%)	279 (65.2%)	5.50E-14	1,081 (92.7%)	0 (0.00%)	7.90E-181	802 (94.2%)	279 (88.6%)	3.60E-03	0 (0.00%)	0 (0.00%)	1

HPV-18	58 (4.2%)	40 (4.2%)	18 (4.2%)	1	58 (5.0%)	0 (0.00%)	3.60E-04	40 (4.7%)	18 (5.7%)	0.63	0 (0.00%)	0 (0.00%)	1
HPV 6/11(+)(low risk strains)	32 (2.3%)	11 (1.2%)	21 (4.9%)	1.80E-04	32 (2.7%)	0 (0.00%)	1.50E-02	11 (1.3%)	21 (6.7%)	4.10E-05	0 (0.00%)	0 (0.00%)	1
HPV-6	23 (1.7%)	7 (0.74%)	16 (3.7%)	3.60E-04	23 (2.0%)	0 (0.00%)	0.063	7 (0.82%)	16 (5.1%)	7.00E-05	0 (0.00%)	0 (0.00%)	1
HPV-11	9 (0.65%)	4 (0.42%)	5 (1.2%)	0.29	9 (0.77%)	0 (0.00%)	0.41	4 (0.47%)	5 (1.6%)	0.13	0 (0.00%)	0 (0.00%)	1
Tumor mutational burden (TMB) TMB≥10mutations/ Mb	244 (17.7%)	171 (18.0%)	73 (17.1%)	0.94	214 (18.3%)	30 (14.0%)	0.29	156 (18.3%)	58 (18.4%)	1	15 (14.8%)	15 (13.3%)	1
Microsatellite instability (based on evaluable cases)	12 (0.89%)	10 (1.1%)	2 (0.48%)	0.69	8 (0.71%)	4 (1.9%)	0.22	7 (0.84%)	1 (0.33%)	0.83	3 (3.0%)	1 (0.90%)	0.92

Table 2: Immunological profile of ASCC based on sex and HPV status.

	OVERALL							**HPV(+)**			**HPV(-)**		
Cohort	**ASCC (all) (N=1,380)**	**Female (N=952)**	**Male (N=428)**	**P value (Female vs. Male)**	**HPV(+) (N=1,166)**	**HPV(-) (N=214)**	**P value (HPV(+) vs. HPV(-))**	**Female HPV(+) (N=851)**	**Male HPV(+) (N=315)**	**P value (Female vs. Male)**	**Female HPV(-) (N=101)**	**Male HPV(-) (N=113)**	**P value (Female vs. Male)**
Evaluable for PD-L1(+)	464	312	152		392	72		282	110		30	42	
PD-L1(+) (1%+)	338 (72.8%)	231 (74.0%)	107 (70.4%)	0.8	292 (74.5%)	46 (63.9%)	0.16	213 (75.5%)	79 (71.8%)	1	18 (60.0%)	28 (66.7%)	0.83
PD-L1 High(+) (50%+)	91 (19.6%)	59 (18.9%)	32 (21.1%)	0.8	74 (18.9%)	17 (23.6%)	0.34	55 (19.5%)	19 (17.3%)	1	4 (13.3%)	13 (31.0%)	0.74
PD-L1 Low (1-49%)(+)	247 (53.2%)	172 (55.1%)	75 (49.3%)	0.8	218 (55.6%)	29 (40.3%)	0.054	158 (56.0%)	60 (54.5%)	1	14 (46.7%)	15 (35.7%)	0.74
Evaluable for Tumor Mutational Burden (TMB)	1,380	952	428		1166	214		851	315		101	113	
TMB-High (10+ mutations/Mb)	244 (17.7%)	171 (18.0%)	73 (17.1%)	0.8	214 (18.4%)	30 (14.0%)	0.16	156 (18.3%)	58 (18.4%)	1	15 (14.9%)	15 (13.3%)	0.84
TMB-Low (< 10 mutations/ Mb)	1,136 (82.3%)	781 (82.0%)	355 (82.9%)	0.8	952 (81.6%)	184 (86.0%)	0.16	695 (81.7%)	257 (81.6%)	1	86 (85.1%)	98 (86.7%)	0.84
Evaluable for Microsatellite Instability	1,344	930	414		1134	210		831	303		99	111	
MSI-High	12 (0.89%)	10 (1.1%)	2 (0.48%)	0.8	8 (0.71%)	4 (1.9%)	0.16	7 (0.84%)	1 (0.33%)	1	3 (3.0%)	1 (0.90%)	0.74
MSS	1,319 (98.1%)	912 (98.1%)	407 (98.3%)	0.83	1,119 (98.7%)	200 (95.2%)	2.10E-02	820 (98.7%)	299 (98.7%)	1	92 (92.9%)	108 (97.3%)	0.74
MSI-Low	13 (0.97%)	8 (0.86%)	5 (1.2%)	0.8	7 (0.62%)	6 (2.9%)	3.60E-02	4 (0.48%)	3 (0.99%)	1	4 (4.0%)	2 (1.8%)	0.74

Table 3: Prevalence of select gene alterations based on sex and HPV status in ASCC.

	OVERALL							HPV(+)			HPV(-)		
Cohort	**ASCC (all) (N=1,380)**	**Female (N=952)**	**Male (N=428)**	**P value (Female vs. Male)**	**HPV(+) (N=1,166)**	**HPV(-) (N=214)**	**P value (HPV(+) vs. HPV(-))**	**Female HPV(+) (N=851)**	**Male HPV(+) (N=315)**	**P value (Female vs. Male)**	**Female HPV(-) (N=101)**	**Male HPV(-) (N=113)**	**P value (Female vs. Male)**
PIK3CA	469 (34.0%)	337 (35.4%)	132 (30.8%)	0.24	433 (37.1%)	36 (16.8%)	2.60E-08	316 (37.1%)	117 (37.1%)	1	21 (20.8%)	15 (13.3%)	0.46
KMT2D	256 (18.6%)	200 (21.0%)	56 (13.1%)	3.50E-03	235 (20.2%)	21 (9.8%)	1.30E-03	186 (21.9%)	49 (15.6%)	0.16	14 (13.9%)	7 (6.2%)	0.38
FBXW7	185 (13.4%)	129 (13.6%)	56 (13.1%)	0.97	164 (14.1%)	21 (9.8%)	0.18	117 (13.7%)	47 (14.9%)	0.78	12 (11.9%)	9 (8.0%)	0.89
PTEN	182 (13.2%)	146 (15.3%)	36 (8.4%)	3.50E-03	168 (14.4%)	14 (6.5%)	4.70E-03	136 (16.0%)	32 (10.2%)	0.15	10 (9.9%)	4 (3.5%)	0.43
TP53	161 (11.7%)	74 (7.8%)	87 (20.3%)	1.70E-09	65 (5.6%)	96 (44.9%)	4.10E-43	44 (5.2%)	21 (6.7%)	0.59	30 (29.7%)	66 (58.4%)	8.20E-04
SOX2	119 (8.6%)	81 (8.5%)	38 (8.9%)	0.97	109 (9.3%)	10 (4.7%)	0.07	77 (9.0%)	32 (10.2%)	0.78	4 (4.0%)	6 (5.3%)	0.99
TERC	112 (8.1%)	77 (8.1%)	35 (8.2%)	1	103 (8.8%)	9 (4.2%)	0.064	74 (8.7%)	29 (9.2%)	0.89	3 (3.0%)	6 (5.3%)	0.91
FGF12	109 (7.9%)	78 (8.2%)	31 (7.2%)	0.82	96 (8.2%)	13 (6.1%)	0.42	73 (8.6%)	23 (7.3%)	0.78	5 (5.0%)	8 (7.1%)	0.96
PRKCI	101 (7.3%)	66 (6.9%)	35 (8.2%)	0.68	93 (8.0%)	8 (3.7%)	0.08	63 (7.4%)	30 (9.5%)	0.55	3 (3.0%)	5 (4.4%)	0.99
TERT	98 (7.1%)	33 (3.5%)	65 (15.2%)	5.90E-12	31 (2.7%)	67 (31.3%)	5.40E-34	16 (1.9%)	15 (4.8%)	0.15	17 (16.8%)	50 (44.2%)	8.10E-04
CDKN2A	96 (7.0%)	36 (3.8%)	60 (14.0%)	1.40E-09	34 (2.9%)	62 (29.0%)	4.00E-29	20 (2.4%)	14 (4.4%)	0.38	16 (15.8%)	46 (40.7%)	1.20E-03
EP300	92 (6.7%)	65 (6.8%)	27 (6.3%)	0.97	83 (7.1%)	9 (4.2%)	0.23	60 (7.1%)	23 (7.3%)	0.96	5 (5.0%)	4 (3.5%)	0.99
NOTCH1	90 (6.5%)	51 (5.4%)	39 (9.1%)	4.90E-02	59 (5.1%)	31 (14.5%)	2.80E-05	37 (4.3%)	22 (7.0%)	0.38	14 (13.9%)	17 (15.0%)	1
STK11	78 (5.7%)	61 (6.4%)	17 (4.0%)	0.22	67 (5.7%)	11 (5.1%)	0.93	53 (6.2%)	14 (4.4%)	0.59	8 (7.9%)	3 (2.7%)	0.46
KDM6A	69 (5.0%)	39 (4.1%)	30 (7.0%)	0.11	59 (5.1%)	10 (4.7%)	1	38 (4.5%)	21 (6.7%)	0.48	1 (0.99%)	9 (8.0%)	0.21
RB1	68 (4.9%)	49 (5.1%)	19 (4.4%)	0.91	47 (4.0%)	21 (9.8%)	4.10E-03	37 (4.3%)	10 (3.2%)	0.67	12 (11.9%)	9 (8.0%)	0.89
FGF3	65 (4.7%)	34 (3.6%)	31 (7.2%)	2.10E-02	45 (3.9%)	20 (9.3%)	4.70E-03	28 (3.3%)	17 (5.4%)	0.47	6 (5.9%)	14 (12.4%)	0.46
CASP8	64 (4.6%)	40 (4.2%)	24 (5.6%)	0.46	43 (3.7%)	21 (9.8%)	2.60E-03	35 (4.1%)	8 (2.5%)	0.53	5 (5.0%)	16 (14.2%)	0.3
CREBBP	63 (4.6%)	52 (5.5%)	11 (2.6%)	0.062	48 (4.1%)	15 (7.0%)	0.15	42 (4.9%)	6 (1.9%)	0.16	10 (9.9%)	5 (4.4%)	0.5
FGF4	63 (4.6%)	33 (3.5%)	30 (7.0%)	2.30E-02	43 (3.7%)	20 (9.3%)	4.10E-03	27 (3.2%)	16 (5.1%)	0.5	6 (5.9%)	14 (12.4%)	0.46
FGF19	61 (4.4%)	31 (3.3%)	30 (7.0%)	1.70E-02	41 (3.5%)	20 (9.3%)	2.60E-03	25 (2.9%)	16 (5.1%)	0.47	6 (5.9%)	14 (12.4%)	0.46
CCND1	60 (4.3%)	30 (3.2%)	30 (7.0%)	1.70E-02	40 (3.4%)	20 (9.3%)	2.40E-03	24 (2.8%)	16 (5.1%)	0.38	6 (5.9%)	14 (12.4%)	0.46
NFE2L2	55 (4.0%)	31 (3.3%)	24 (5.6%)	0.16	52 (4.5%)	3 (1.4%)	0.084	29 (3.4%)	23 (7.3%)	0.15	2 (2.0%)	1 (0.88%)	0.97
DNMT3A	51 (3.7%)	41 (4.3%)	10 (2.3%)	0.22	46 (3.9%)	5 (2.3%)	0.42	38 (4.5%)	8 (2.5%)	0.51	3 (3.0%)	2 (1.8%)	0.99
FGFR3	51 (3.7%)	41 (4.3%)	10 (2.3%)	0.22	43 (3.7%)	8 (3.7%)	1	36 (4.2%)	7 (2.2%)	0.47	5 (5.0%)	3 (2.7%)	0.91
ASXL1	49 (3.6%)	33 (3.5%)	16 (3.7%)	0.97	39 (3.3%)	10 (4.7%)	0.42	27 (3.2%)	12 (3.8%)	0.78	6 (5.9%)	4 (3.5%)	0.91
CDKN2B	44 (3.2%)	21 (2.2%)	23 (5.4%)	2.10E-02	19 (1.6%)	25 (11.7%)	1.90E-09	11 (1.3%)	8 (2.5%)	0.52	10 (9.9%)	15 (13.3%)	0.91
NFKBIA	42 (3.0%)	28 (2.9%)	14 (3.3%)	0.94	34 (2.9%)	8 (3.7%)	0.63	27 (3.2%)	7 (2.2%)	0.69	1 (0.99%)	7 (6.2%)	0.38
FAS	41 (3.0%)	33 (3.5%)	8 (1.9%)	0.26	39 (3.3%)	2 (0.93%)	0.15	32 (3.8%)	7 (2.2%)	0.55	1 (0.99%)	1 (0.88%)	1
PIK3R1	40 (2.9%)	28 (2.9%)	12 (2.8%)	1	37 (3.2%)	3 (1.4%)	0.29	27 (3.2%)	10 (3.2%)	1	1 (0.99%)	2 (1.8%)	1
BRCA1	39 (2.8%)	22 (2.3%)	17 (4.0%)	0.24	36 (3.1%)	3 (1.4%)	0.37	21 (2.5%)	15 (4.8%)	0.38	1 (0.99%)	2 (1.8%)	1
BAP1	37 (2.7%)	28 (2.9%)	9 (2.1%)	0.69	31 (2.7%)	6 (2.8%)	0.89	26 (3.1%)	5 (1.6%)	0.53	2 (2.0%)	4 (3.5%)	0.99
ARID1A	35 (2.5%)	25 (2.6%)	10 (2.3%)	0.97	26 (2.2%)	9 (4.2%)	0.18	18 (2.1%)	8 (2.5%)	0.78	7 (6.9%)	2 (1.8%)	0.43
CD274	35 (2.5%)	28 (2.9%)	7 (1.6%)	0.39	34 (2.9%)	1 (0.47%)	0.08	28 (3.3%)	6 (1.9%)	0.53	0 (0.00%)	1 (0.88%)	1
NOTCH3	34 (2.5%)	24 (2.5%)	10 (2.3%)	1	26 (2.2%)	8 (3.7%)	0.33	19 (2.2%)	7 (2.2%)	1	5 (5.0%)	3 (2.7%)	0.91
AKT2	33 (2.4%)	25 (2.6%)	8 (1.9%)	0.68	32 (2.7%)	1 (0.47%)	0.11	25 (2.9%)	7 (2.2%)	0.8	0 (0.00%)	1 (0.88%)	1
MYC	33 (2.4%)	15 (1.6%)	18 (4.2%)	2.70E-02	23 (2.0%)	10 (4.7%)	0.073	14 (1.6%)	9 (2.9%)	0.53	1 (0.99%)	9 (8.0%)	0.21
PDCD1LG2	33 (2.4%)	26 (2.7%)	7 (1.6%)	0.46	31 (2.7%)	2 (0.93%)	0.25	25 (2.9%)	6 (1.9%)	0.67	1 (0.99%)	1 (0.88%)	1
SPEN	33 (2.4%)	26 (2.7%)	7 (1.6%)	0.46	28 (2.4%)	5 (2.3%)	1	23 (2.7%)	5 (1.6%)	0.67	3 (3.0%)	2 (1.8%)	0.99
CBFB	32 (2.3%)	31 (3.3%)	1 (0.23%)	1.80E-03	31 (2.7%)	1 (0.47%)	0.11	30 (3.5%)	1 (0.32%)	4.30E-02	1 (0.99%)	0 (0.00%)	0.91
BRCA2	31 (2.2%)	18 (1.9%)	13 (3.0%)	0.46	26 (2.2%)	5 (2.3%)	0.89	18 (2.1%)	8 (2.5%)	0.78	0 (0.00%)	5 (4.4%)	0.38
KRAS	31 (2.2%)	23 (2.4%)	8 (1.9%)	0.91	24 (2.1%)	7 (3.3%)	0.42	19 (2.2%)	5 (1.6%)	0.78	4 (4.0%)	3 (2.7%)	0.99

EGFR	29 (2.1%)	15 (1.6%)	14 (3.3%)	0.19	22 (1.9%)	7 (3.3%)	0.3	13 (1.5%)	9 (2.9%)	0.5	2 (2.0%)	5 (4.4%)	0.91
AKT1	28 (2.0%)	15 (1.6%)	13 (3.0%)	0.23	23 (2.0%)	5 (2.3%)	0.89	14 (1.6%)	9 (2.9%)	0.53	1 (0.99%)	4 (3.5%)	0.89
CTNNB1	28 (2.0%)	20 (2.1%)	8 (1.9%)	0.97	23 (2.0%)	5 (2.3%)	0.89	18 (2.1%)	5 (1.6%)	0.78	2 (2.0%)	3 (2.7%)	1
TGFBR2	27 (2.0%)	21 (2.2%)	6 (1.4%)	0.65	24 (2.1%)	3 (1.4%)	0.89	20 (2.4%)	4 (1.3%)	0.63	1 (0.99%)	2 (1.8%)	1
APC	26 (1.9%)	18 (1.9%)	8 (1.9%)	1	20 (1.7%)	6 (2.8%)	0.38	14 (1.6%)	6 (1.9%)	0.89	4 (4.0%)	2 (1.8%)	0.91
JAK2	25 (1.8%)	19 (2.0%)	6 (1.4%)	0.74	24 (2.1%)	1 (0.47%)	0.26	19 (2.2%)	5 (1.6%)	0.78	0 (0.00%)	1 (0.88%)	1
KEAP1	25 (1.8%)	15 (1.6%)	10 (2.3%)	0.64	18 (1.5%)	7 (3.3%)	0.18	12 (1.4%)	6 (1.9%)	0.78	3 (3.0%)	4 (3.5%)	1
NOTCH2	25 (1.8%)	17 (1.8%)	8 (1.9%)	1	15 (1.3%)	10 (4.7%)	8.40E-03	12 (1.4%)	3 (0.95%)	0.88	5 (5.0%)	5 (4.4%)	1

(Only gene altered in at least 25 cases are shown.)

5. Discussion

Within our cohort, we observed differences in the prevalence of HPV based on sex. Notably, HPV-16 is identified more frequently in female patients, whereas HPV-18 seems to be similarly prevalent in the male and female patients. Interestingly, though relatively rarer, HPV-6(+) incidence was significantly higher in male patients. Male patients also had higher rates of alterations for TERT (15.2% vs. 3.5%), TP53 (20.3% vs. 7.8%), and CDKN2A (14.0% vs. 3.8%), among other genes. When further examining sex-based differences in HPV(+) cases, we generally did not observe statistically significant differences in gene alteration prevalence, with the exception of CBFB; alterations in CBFB were nearly exclusively found in female HPV(+) patients.

The higher incidence of HPV positivity in women compared to men (nearly double) remains the main hypothesis to justify the better prognosis of ASCC in women. In fact, HPV is a DNA virus with an important role in ASCC development, by inhibiting the tumor suppressor proteins p53 and retinoblastoma (pRb) via viral oncoproteins E6 and E7, which may confer greater sensitivity to chemotherapy and radiotherapy [14]. HPV may prolong G2 cell cycle arrest and increase apoptosis following radiation exposure in addition to affecting DNA repair capacity. The loss of p53 function, more frequently associated with men exhibiting HPV negative ASCC, has been linked to inferior treatment response and resistance to radiotherapy [15]. Meulendijks et al, reported p53 mutations in 80% of HPV negative ASCC compared to 6% in HPV positive cases that had better OS and regional control [16]. This difference in prevalence of alterations affecting p53 is also observed in our study. Specifically, in HPV negative population, 60% of male patients exhibited a TP53 alteration, compared to 30% of female patients. Additionally, in the general population of ASCC, we observed a higher prevalence of TP53 alterations in male patients (20.3%) compared to female patients (7.8%). These data could help to justify the different prognosis reported in the literature.

Some studies used p16 expression as a marker to evaluate HPV status, in addition to HPV DNA, to improve detection of HPV infection and assessment of prognosis. p16 is a protein that regulates cell cycle and has a tumor suppressor effect. p16 expression is repressed by the Rb protein, which is inhibited by HPV, resulting in overexpression of p16. Overexpression of p16 has therefore been widely used as a surrogate marker for transforming HPV infection. However, HPV DNA can be found without transforming relevance and hence without p16 overexpression [16]. In fact, p16 overexpression without HPV association is commonly observed. This could be explained by oncogenic stress such as tobacco and alcohol in head and neck cancer, or through parallel mutations which may compromise downstream (Rb) pathway. RB1 alterations were relatively rare in our cohort (4.9%). However, RB1 alterations were reported in 10% of HPV negative cases compared to only 4% of HPV positive cases ($p < 10\text{-}2$), thus suggesting a possible role in the pathogenesis of this disease within this subgroup of patients. The carcinogenesis of ASCC is so strongly correlated to HPV and p16 pathway [17], and the combined evaluation of both HPV and p16 status in ASCC seems to prognostically indicate the impact of viral transformation on the disease course: identifying extremely good risk (HPV+/p16+) and extremely poor risk (HPV-/p16+, HPV-/p16-) patient cohorts [18]. This observation needs to be validated prospectively in future studies, since it may have a possible clinical impact through an attempt to reduce the intensity of treatment in patients considered to be high risk and consequently to intensify HPV vaccination campaign low instead of high risk [19].

Genomic profiling of ASCC examined in this study also sheds light on potential targets for new therapeutic options in this disease. Approximately 30% of patients, regardless of sex and HPV expression have alteration of PIK3CA, that potentially makes this disease eligible to treatment with targeted tyrosine kinase inhibitors [20,21]. Few pre-clinical studies report that PI3K/mTOR inhibition prevents anal carcinogenesis in a human papillomavirus model of anal cancer [22,23] but no clinical data on PI3K inhibitor in anal cancer is available in the literature. Cacheux et al. [24] found a 20% of PIK3CA alteration in 148 ASCC; the distribution of mutation was similar between treatment naïve tumor and recurrence; PIK3CA status retained its prognostic significance in Cox multivariate regression analysis. Again, Myeong-Kyun S et al. demonstrated that activating mutations in PIK3CA drive anal

carcinogenesis together with HP16 oncogenes and that PI3K/mTOR pathway is a relevant target for therapeutic intervention [25]. Alpelisib is a PIK3CA inhibitor, administered with fulvestrant, prolonged progression-free survival (20 vs 11 months) and improved response rate (26.6% vs. 12.8%) among patients with PIK3CA-mutated, HR-positive, HER2-negative advanced breast cancer who had received endocrine therapy previously [26]. Additionally, a large population of ASCC appears to have an immunological profile, susceptible to immunotherapy. Over 70% of the population was positive for PD-L1 (1%+), with nearly 20% exhibiting a high PDL-1 expression (50%+). Cemiplimab and pembrolizumab monotherapy significantly improved overall survival and progression-free survival compared with chemotherapy in patients with advanced non-small-cell lung cancer with PD-L1 of at least 50%, providing a new treatment option for this patient population avoiding chemotherapy treatment and improving patients' quality of life [27,28]. Again, ~18% of the cases had a high tumor mutational burden (TMB>=10 mutations/Mb), predictive of response to immunotherapy [29]. Recently, the FDA has approved pembrolizumab in all cancers with a TMB > 10Mut/Mb based on findings from the phase 2 KEYNOTE-158 [30]. A few clinical trials exploring the activity of pembrolizumab, nivolumab and avelumab after the first line setting, report an interesting response rate of 10-24%, a median PFS of 2-4.1 months and a median OS of 9.3-13.9 months [31-33]. A better response was observed in patients with a PDL-1 positivity which represent about 40% of patient population [31]. Interestingly, ~5% of patients (n=67) have a BRCA1/2 alteration with a subset of these cases (n=21 out of the 51 evaluable cases) showing evidence for biallelic inactivation of BRCA1/2; this may provide a new therapeutic horizon for this small subgroup of patients, since these mutations predict platinum sensitivity and PARP inhibitor activity] as demonstrated in many diseases as ovarian, breast and prostatic cancer [34, 35]. To our knowledge, this is the largest genetic profiling study in ASCC to date. We sought to justify the different prognosis based on sex in ASCC by examining the genetic profiles of a large number of ASCC patients. Findings from this large cohort seem to confirm the prognostic role of HPV infection on ASSC patients excluding a role of cancer genes. Again, possible new therapeutic hypotheses seem to emerge in a disease with a favorable prognosis but few therapeutic options available.

6. Funding

This research received no external funding.

7. Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

8. Data Availability Statement

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

9. Acknowledgment

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10. Conflicts of Interest

All the authors declare no conflict of interest.

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