SHORT REPORT

Genomic analysis of HPV-positive versus HPV-negative oesophageal adenocarcinoma identifies a differential mutational landscape

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ABSTRACT

Background High-risk human papillomavirus (hr-HPV) has been implicated in a subset of patients with oesophageal adenocarcinoma (OAC). We therefore hypothesised that HPV associated OAC may have distinct genomic aberrations compared with viral negative oesophageal cancer.

Methods Whole exome sequencing was performed to explore the mutational landscape and potential molecular signature of HPV-positive versus HPV-negative OAC. Four hr-HPV-positive and 8 HPV-negative treatment-naive fresh-frozen OAC tissue specimens and matched normal tissue were analysed to identify somatic genomic mutations. Data were subjected to cancer driver gene identification and pathway analysis.

Results The HPV-positive cohort harboured approximately 50% less non-silent somatic mutations than the virus-negative patients with oesophageal cancer (1.31 mutations/Mb vs 2.56 mutations/Mb, p=0.048). TP53 aberrations were absent in the HPV-positive OAC group whereas 50% of the HPV-negative patients with OAC exhibited TP53 mutations. HPV-negative cancers were enriched with non-silent mutations in cancer driver genes, but not HPV-positive tumours. Enriched A>C transversions at adenine-adenine (AA) dinucleotide was observed in 5/7 Siewert class I OAC samples but none (0/5) in Siewert class II tumours (p=0.027).

Conclusions These findings demonstrate distinct genomic differences between HPV-positive and HPV-negative OACs indicating different biological mechanisms of tumour formation.

INTRODUCTION

Oesophageal adenocarcinoma (OAC) has been one of the fastest growing cancers in the Western world and the reason(s) for this are obscure. Our discovery that high risk human papillomavirus (hr-HPV) is strongly associated with a subset of patients with Barrett's dysplasia (BD) (a premalignant lesion) and OAC may be one of the 'missing' strong risk factors responsible for the significant rise of this malignancy since the 1970s, as has been the case with head and neck squamous cell carcinoma (HNSCC), another virus associated cancer.[1 2] In fact, hr-HPV has now been proposed as a risk factor for OAC.[3] Nevertheless, prior negative studies exist due to poor tissue classification or suboptimal testing methodology as well as racial and geographical variations.[4 5]

Whole genome sequencing and whole exome sequencing (WES) studies have revealed that somatic mutations in OAC are markedly heterogeneous,[6] highlighting diverse carcinogenic pathways. Significantly, the tumour suppressor genes, TP53 and CDKN2A (P16INK4a) are recurrently targeted in OAC.[6 7] TP53 is mutated in over 50% of OAC's and CDKN2A in 12%.[6 7]

Recently, we have reported that persistently detectable dysplasia/neoplasia after endoscopic ablative treatment of BD/OAC was associated with HPV persistence or p53 overexpression. Furthermore, we have demonstrated for the first time that BD/intramucosal OAC samples positive for transcriptional markers of HPV activity were mostly devoid of p53 overexpression (84%).[8] Next generation sequencing revealed that the vast majority of biologically active hr-HPV tumours had wild type TP53;[8] one of the hallmarks of HPV driven cervical and head and neck malignancies.[9 10]

Compared with HPV-negative HNSCC, HPV-positive cancers have fewer DNA copy number alterations, less genome-wide hypomethylation, less frequent or absent TP53 mutations (due to hr-HPV E6 oncoprotein induced degradation of p53), fewer gene mutations and lower expression of epidermal growth factor receptor (EGFR).[10–13]

Therefore, we hypothesised that HPV associated OAC may have a distinct distribution of molecular aberrations and genomic abnormalities compared with HPV-negative oesophageal cancer, which may indicate different pathways to carcinogenesis. Thus, we analysed the genomic landscape of HPV-positive versus virus-negative OAC to ascertain if there was a unique molecular signature that differentiated these tumours.

MATERIALS AND METHODS

Patient and tumour characteristics

Twelve fresh-frozen, treatment-naïve resected specimens of OAC (Siewert class I, n=7; Siewert Class II, n=5) were subjected to WES. Tissue dissection was performed to enrich cancer content to over 70%. HPV status and E6/E7 oncogene activity was determined by nested PCR and RT-PCR detection of E6/7 transcripts described previously.[1] Oncogene activity was further evaluated by p16INK4A immunohistochemistry (IHC), a surrogate marker for hr-HPV E7 oncoprotein.[14]

Exome sequencing and variant detection

WES was performed at the Ramaciotti Centre, University of New South Wales, Sydney using the

Illumina HiSeq2000 platform. The data analysis workflow is illustrated in online supplementary figure S1. Details of WES analysis, identification of recurrently mutated genes, cancer driver gene classification, viral integration analysis and mutation validation are available in the online supplementary methods section.

Statistical assessment

The non-parametrical, Mann-Whitney U test was used to assess the difference in mutational frequency between HPV-positive and HPV-negative groups. Fisher's exact test was used to compare the frequency of the enriched A>C transversions at AA dinucleotide in the Siewert Class I versus Class II tumours. Additionally, Fisher's exact test was used to assess the statistical enrichment of mutated cancer driver genes in the HPV-positive and HPV-negative groups separately. $p<0.05$ was considered statistically significant.

RESULTS

Patient and tumour characteristics

Patient and tumour characteristics are shown in online supplementary table S1. Among 12 patients, 4 individuals were classified within the HPV-positive group as their tumours were positive for hr-HPV DNA, E6/E7 mRNA (RT-PCR) and p16 INK4A immunohistochemistry. In the remaining eight patients, six were negative for HPV DNA and viral oncogene transcriptional markers (E6/7 mRNA plus p16INK4A) whereas the other two had only detectable HPV DNA but without biological activity. Thus, to be included in the HPV-positive group, viral transcriptional markers had to be detectable in addition to HPV DNA. The median age of the HPV-positive group was 55 years and the virus-negative group, 67 years.

WES analysis

Somatic mutations in OAC were identified using WES on 12 matched tumour-normal sample pairs. Average coverage of 99.3X (range: 79–122X) and 82.5X (range: 62–106X) was achieved for the target regions of the tumour and the normal samples, respectively (see online supplementary table S2). A total of 6936 somatic mutations including 6860 single nucleotide variations (SNVs) and 76 small insertions/deletions (indels) were identified across the cohort, in which there were 1580 non-silent mutations (see online supplementary tables S2 and S3) and 407 synonymous mutations. The median frequency of non-silent mutations was 126 (range of 47–212) mutations/Mb or 2.045 (range of 0.69–3.44) mutations/Mb (see online supplementary table S2), and HPV-positive samples harboured significantly less non-silent mutations than HPV-negative samples (1.31 mutations/Mb vs 2.56 mutations/Mb, p value=0.048) (figure 1A).

Mutational signatures

We analysed the spectrum of 1975 SNVs in the encoding region and observed a high prevalence of C>A transversions, which comprised an average of 53% of the total mutations in the encoding region (see online supplementary table S4). We found no difference in frequency of C>A transversions between HPV-positive and HPV-negative samples. Although the overall frequency of A>C transversions (or T>G transversions on the complementary strand) was 9% in the encoding region, this mutational pattern (at AA dinucleotides) was enriched (ie, mutation frequency >5%) in five of seven Siewert class I OAC samples (A, D, E, I and J, figure 1B and see online supplementary figure S2). Four of these samples coexisted with Barrett's oesophagus (BO). This mutational signature was not observed in any of the Siewert class II tumour samples (n=5), only one of which had associated BO (p=0.027). No enrichment of mutations at Cs in TpC context (Cs with a T on their 5′ side) was found in HPV-positive samples compared with HPV-negative specimens.

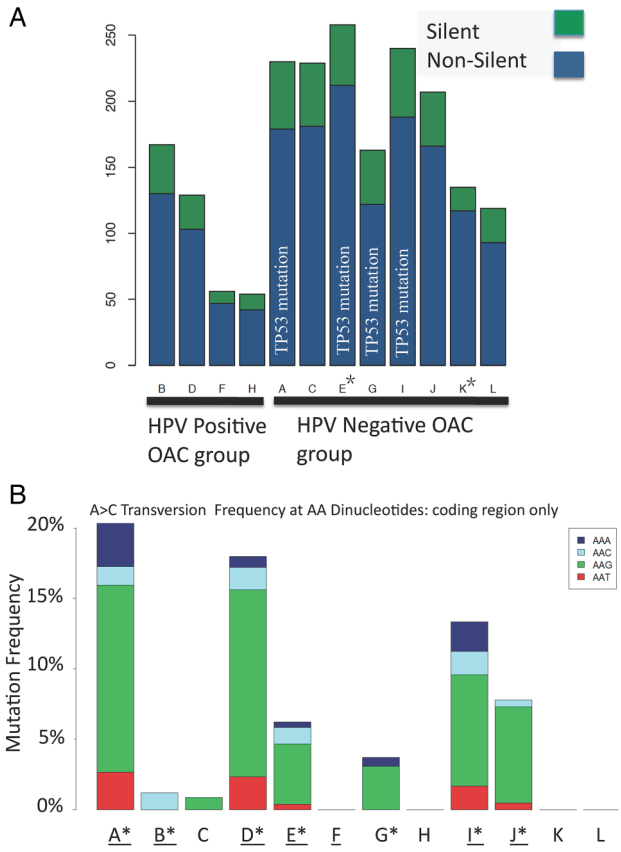

Figure 1 (A) The number of silent and non-silent somatic mutations in each patient. *(Patients E (TP53 mutated) and K (wild type TP53)) indicates the samples with HPV DNA positivity but without viral oncogene E6/E7 activity. (B) The frequency of AA>AC transversions as a percentage of total mutations in the coding region in 12 patients. The stacked bars indicate the 3-primer nucleotide. *Represents Siewert Class I cancers while the rest were Siewert Class II tumours. Tumour samples that coexisted with Barrett's oesophagus (BO) are underlined. HPV, human papillomavirus; OAC, oesophageal adenocarcinoma.

Cancer driver genes and pathways

Among 1376 genes containing non-silent mutations, 47 genes were considered as potential drivers of carcinogenesis (see online supplementary tables S5 and S6). Ten cancer driver genes were observed in HPV-positive tumours and 38 genes in HPV-negative cancer lesions (one gene, translocated promoter region, nuclear basket protein (TPR) was mutated in both groups). Significantly, more than the expected number of cancer driver genes were mutated in HPV-negative cancers (p=0.026). In contrast, there was no such difference in the HPV-positive tumours (p=0.34).

Classification of diverse cancer driver genes into signalling pathways that control fundamental cellular processes revealed that DNA damage control (8/12), chromatin modification (7/12) and cell cycle/apoptosis (7/12) pathways were the most frequently affected (table 1).

Virus genome integration analysis

Virus genome integration analysis revealed that the most commonly integrated virus was the human endogenous retrovirus

Table 1 List of cancer driver genes in human papillomavirus (HPV)-positive and HPV-negative tumour groups classified into 12 pathways that control three fundamental cellular processes

	Cell survival					Cell fate				Genome maintenance
	TGF-β	MAPK	STAT	P13K	RAS	Cell cycle/ apoptosis	Notch	HH	APC	Chromatin modification	Transcriptional regulation	DNA damage control
HPV+												
Patient B										CREBBP/ARID1B	CREBBP	MUTYH
Patient D												STAG2
Patient F						TPR/CHEK2						CHEK2
Patient H			MPL									
HPV−												
Patient A		FGFR1		FGFR1	FGFR1	TP53 CCND2				MLL3		TP53
Patient C	SMAD4		IL6ST	ALK/TSC2	ALK	TSC2				WHSC1L1		TSC2
Patient E						TP53				ASXL1		TP53/BRCA2
Patient G						TP53 /TPR			CTNNB1	DNMT1/SET	AR	TP53
Patient I						TP53/CASP8				KDM6A		TP53/FANCA
Patient J										SMARCB1	MLLT3	
Patient K		TSHR		TSHR	CIC	PPP2R1A						
Patient L							MAML2	SMO				

*Only 32/47 cancer driver genes are included in the table as the pathways of the remaining 15 genes (2 mutated in the HPV-positive group, 13 mutated in HPV-negative group) could not be characterised according to Vogelstein's publication (reference 6, supplementary methods and results), Cancer Gene Census, kyoto encyclopedia of genes and genomes (KEGG) and Reactome databases.
ALK, anaplastic lymphoma kinase; APC, adenomatous polyposis coli; AR, androgen receptor; CIC, capicua homolog; CREBBP, CREB (cAMP-response element binding protein) binding protein; FANCA, fanconi anemia, complementation group A; HH, hedgehog; MAPK, mitogen-activated protein kinases; MPL, myeloproliferative leukemia virus oncogene; RAS, ras genes; SET, SET nuclear proto-oncogene; SMO, smoothened; TSHR, thyroid stimulating hormone receptor.

type K. In two out of four samples positive for hr-HPV DNA and E6/E7 oncogenic activity, the integrated form of HPV16 was identified in a number of discordant pairs (one read mapped to the human genome, while the other read mapped to the viral genome). The integrated viral genome fragment sizes varied between 20 bp and 60 bp.

Mutation validation

In sixty-nine randomly selected SNVs identified from WES, 55 mutations were successfully confirmed by Sanger sequencing. Wild type and indel-specific PCR verified seven of eight small indels whereas one of eight could not be confirmed as the insertion was the result of sequencing duplication (see online supplementary table S7). Overall 62/77 (80.5%) mutations were confirmed.

DISCUSSION

Our data has revealed for the first time a distinct distribution of molecular genomic abnormalities in HPV-positive OAC as opposed to virus-negative oesophageal cancer. The HPV-positive OAC group harboured approximately 50% less non-silent mutations than the HPV-negative oesophageal cancer samples. These findings are similar to a WES study of HNSCC (another HPV driven cancer) where the mutation rate of HPV-positive cancers was approximately half that of virus-negative tumours (2.28 mutations/Mb vs 4.83 mutations/Mb).[13]

In another study, head and neck tumours obtained from patients with a smoking history were associated with more mutations than those malignant lesions found in non-smokers. Again, HPV-positive cancers had less mutations than virus-negative HNSCC.[10] Conversely, these findings were not replicated in the more recent Cancer Genome Atlas Study and Seiwert *et al*'s investigation.[15 16] This could be due to the majority of virus-positive patients in the former study being smokers with characteristic tumour cytosine phosphate guanine (CpG) transversions confounding the mutational burden between the groups. Seiwert *et al*'s study population had an unusually high number of HPV-positive cancers (42.5%), which is likely due to the less stringent criteria employed to ascribe a virally driven process. In addition, they only performed targeted sequencing of 617 cancer associated genes.[16]

Significantly, we found that TP53 aberrations were absent in the virus-positive OAC group whereas 50% of the HPV-negative OAC tumours exhibited TP53 mutations. Differences in TP53 mutation frequency between HPV-positive and HPV-negative Barrett's dysplastic and intramucosal OAC tissue samples have also been recently described by us.[8] These data raise the possibility of two distinct mechanisms of disrupting TP53 function in OAC similar to that described for head and neck tumours which are also HPV related.[8 13] One is HPV E6 oncoprotein mediated degradation of TP53 and the other, p53 inactivating mutations.

E6 and E7 oncoproteins expressed by hr-HPV drive malignant transformation and progression in infected epithelia via their interaction with tumour suppressor proteins p53 and Rb, respectively. This leads to genomic instability and cancer development.[17] The expression of these oncoproteins therefore substitutes for multiple oncogenic hits by genetic predilection or environmental mutagens which reduce the number of mutations required to develop carcinoma[18] as revealed in our study.

To date, several studies have reported the enriched A to C transversions at AA sites in OAC,[6 7] and speculated its association with oxidative damage induced by gastro-oesophageal reflux disease.[6] Our findings revealed a similar mutational signature in the majority of Siewert class I tumour patients, most of

whom had coexisting BO. This pattern of changes was absent in class II cancers, almost all of which were devoid of BO. Intriguingly, Dulak also found a similar trend whereby OAC's within the tubular oesophagus exhibited an enrichment of AA transversions compared with junctional tumours. This differential mutational signature characteristic of junctional (Siewert 2) versus tubular oesophageal tumours (Siewert 1) raises the possibility of different carcinogenic processes.

Three common pathways identified in our study cohort, that is, DNA damage control, chromatin modification and cell cycle/apoptosis have been well documented in tumorigenesis. Compromised DNA damage capability and consequent genomic instability are characteristic of many cancers.[19] DNA damage due to acid, bile and smoking have been incriminated in the pathogenesis of OAC. In addition, HPV can damage genes and chromosomes directly.[17] These mutations may trigger oncogene activity or inhibit tumour suppressor function contributing to cancer development in the absence of intact DNA damage control mechanisms. Chromatin-remodelling gene mutations have also been described by Dulak *et al* in a quarter of OACs.[6] Finally, deregulation of cell proliferation and programmed cell death are hallmarks of malignancies.

In our study, viral integration analysis identified hybrid sequences containing HPV16 and the human genome. Notably, in a WES study involving head and neck tumours, HPV16 sequence reads were also detected in a subset of cancers (19%).[13] However, exomes only represent approximately 1–1.5% of the human genome and HPV integration is a random process. Thus, the true extent of viral integration into the human chromosomes can only be revealed by sequencing the entire genome as recently described by Parfenov *et al*.[20] Sequence fragments that contained only viral genome were ignored and not included in the analysis as there was uncertainty about its origin (cell-free/episomal/integrated form of virus).

There are significant clinical implications from these findings. HPV-positive patients with HNSCC have a better response to treatment, lower rate of disease progression as well as a significantly improved overall and disease-specific survival as compared with those with HPV-negative cancers.[14] Similar outcomes may also be true for the role of HPV positivity in OAC. Larger sample size genomic studies are required to confirm our findings. Functional investigations of these mutated genes are also warranted to validate and explain their role in tumour development.

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Contributors SR had full access to all data in the study and takes responsibility for the integrity and the accuracy of the data analysis. Research concept and study design: SR. Experimental work and data acquisition: BW, HCL, JW, SR, NM. Analysis and interpretation of data: SR, BW, HCL, JW, NM, PS and JH. Writing, review and/or revision of the manuscript: SR, BW, HCL, JW, NM, PS and JH.

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