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Activating *KRAS* mutations are characteristic of oncocytic sinonasal papilloma and associated sinonasal squamous cell carcinoma

Aaron M. Udager[1], Jonathan B. McHugh[1], Bryan L. Betz[1], Kathleen T. Montone[2], Virginia A. Livolsi[2], Raja R. Seethala[3], Evgeny Yakirevich[4], O. Hans Iwenofu[5], Bayardo Perez-Ordonez[6], Kathleen E. DuRoss[1], Helmut C. Weigelin[1], Megan S. Lim[2], Kojo S. J. Elenitoba-Johnson[2]*, and Noah A. Brown[1]*

[1]Department of Pathology, University of Michigan Health System, Ann Arbor, MI
[2]Department of Pathology, Perelman School of Medicine at University of Pennsylvania, Philadelphia PA
[3]Department of Pathology, University of Pittsburgh Medical Center, Pittsburgh, PA
[4]Department of Pathology, Brown University, Providence, RI
[5]Department of Pathology, Ohio State University, Columbus, OH
[6]Laboratory Medicine and Pathobiology, University of Toronto, Toronto, CA

Correspondence to:

Noah A. Brown, M.D.
Department of Pathology
University of Michigan Medical School
1107 Traverwood II, Suite C
2910 Huron Parkway
Ann Arbor, MI 48105
Phone: (734) 764-0506
Fax: (734) 764-4102
E-mail: noahbr@umich.edu

Kojo S.J. Elenitoba-Johnson, M.D.
Department of Pathology
University of Pennsylvania Medical School
609A Stellar Chance Laboratories
420 Curie Boulevard
Philadelphia, PA 1904
Phone: (215) 898-8198
Fax: (734) 615-9666
E-mail: Kojo.Elenitoba-Johnson@uphs.upenn.edu

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ABSTRACT

Oncocytic sinonasal papillomas (OSP) are benign tumours of the sinonasal tract, a subset of which are associated with synchronous or metachronous sinonasal squamous cell carcinoma (SNSCC). Activating *EGFR* mutations were recently identified in nearly 90% of inverted sinonasal papillomas (ISP) – a related tumour with distinct morphology. *EGFR* mutations were however not found in OSP, suggesting that different molecular alterations drive the oncogenesis of these tumours. In this study, tissue from 51 cases of OSP and 5 cases of OSP-associated SNSCC was obtained retrospectively from six institutions. Tissue was also obtained from 50 cases of ISP, 22 cases of ISP-associated SNSCC, 10 cases of exophytic sinonasal papilloma (ESP), and 19 cases of SNSCC with no known papilloma association. Using targeted next-generation and conventional Sanger sequencing we identified *KRAS* mutations in 51/51 (100%) OSP and 5/5 (100%) OSP-associated SNSCC. The somatic nature of *KRAS* mutations was confirmed in a subset of cases with matched germline DNA, and four matched pairs of OSP and concurrent associated SNSCC had concordant *KRAS* genotypes. In contrast, *KRAS* mutations were present in only 1 (5%) SNSCC with no known papilloma association and none of the ISP, ISP-associated SNSCC, or ESP. This is the first report of somatic *KRAS* mutations in OSP and OSP-associated SNSCC. The presence of identical mutations in OSP and concurrent associated SNSCC supports the putative role of OSP as a precursor to SNSCC, and the high frequency and specificity of *KRAS* mutations suggests that OSP and OSP-associated SNSCC are biologically distinct from other similar sinonasal tumours. The identification of *KRAS* mutations in all studied

OSP cases represents an important development in our understanding of the pathogenesis of this disease and may have implications for diagnosis and therapy.

INTRODUCTION

Sinonasal (Schneiderian) papillomas, which account for approximately 1-5% of sinonasal tumours, include inverted sinonasal papilloma (ISP; approximately 62%), exophytic sinonasal papilloma (ESP; approximately 32%) and oncocytic sinonasal papilloma (OSP; approximately 6%) [1]. Similar to ISP, OSP usually arises from the lateral nasal wall or paranasal sinuses and is associated with synchronous or metachronous sinonasal squamous cell carcinoma (SNSCC) in a subset of cases (up to 15%). OSP, however, has a distinctive histomorphology comprised of an epithelial proliferation of bland columnar cells with eosinophilic cytoplasm, intraepithelial mucin cysts/neutrophilic abscesses, and mixed exophytic and endophytic growth patterns [1,2]. Importantly, the genetic basis for OSP and associated SNSCC is unknown. Recently, highly-prevalent activating *EGFR* mutations were identified in the vast majority of ISP [3]. These mutations were also commonly present in ISP-associated SNSCC but not in ESP, OSP, or SNSCC without a known papilloma association. These data suggest that the ISP/ISP-associated SNSCC disease spectrum is biologically distinct from other sinonasal lesions, including OSP. Therefore, in this study, we sought to identify possible recurrent oncogenic drivers in OSP and explore the genetic relationship of OSP to associated SNSCC.

MATERIALS AND METHODS

Specimens

With institutional review board approval, formalin-fixed, paraffin-embedded (FFPE) tissue was obtained from six institutions including 51 OSP and 5 OSP-associated SNSCC from 52 unique patients. In addition, FFPE tissue from 50 ISP, 22 ISP-associated SNSCC, 10 ESP, and 19 SNSCC without a known papilloma association was obtained from the University of Michigan, as described previously [3]. All diagnoses were confirmed centrally by an experienced head and neck pathologist (J.B.M.). DNA was extracted using the Pinpoint Slide DNA Isolation System (Zymo Research, Irvine, CA), according to the manufacturer's instructions. For a subset of OSP patients, constitutional DNA was also extracted from adjacent normal sinonasal tissue.

DNA sequencing

Targeted next-generation sequencing was performed as described previously [3]. In brief, sequencing libraries were generated from 10 ng of extracted DNA using the Ion AmpliSeq Cancer Hotspot Panel v2 (Thermo Fisher Scientific, Waltham, MA); variants were called using Torrent Suite 4.0.2 and assessed using the Broad Institute's Integrated Genomics Viewer (IGV 2.3). Conventional bidirectional Sanger sequencing of *KRAS* exons 2 and 3 and *EGFR* exons 18, 19, 20 and 21 was performed using nested sequencing primers, as described previously [3].

RESULTS AND DISCUSSION

In order to identify possible pathogenic mutations, 2 cases of OSP were evaluated using targeted next-generation sequencing. In both cases, a guanine to adenine transition in codon 12, resulting in a glycine to aspartic acid missense substitution (G12D), was identified in the *KRAS* gene (Figure 1). No other mutations in any of the other 49 genes on the Ion AmpliSeq Cancer Hotspot Panel v2 were identified, and in both cases, the *KRAS* G12D mutation was confirmed by Sanger sequencing and demonstrated to be somatic by evaluating constitutional DNA extracted from adjacent normal sinonasal tissue.

To determine the incidence of *KRAS* mutations in OSP and OSP-associated SNSCC, we evaluated a large, multi-institutional cohort of tumours by Sanger sequencing of exons 2 and 3 (Supplementary Table 1). Overall, 100% of OSP (n = 51) harboured *KRAS* mutations, either in codon 12 or 61 (Figure 2). Importantly, all identified mutations have previously been described in a number of common malignancies including colon, pancreas and lung cancer [4-6]. In addition, all mutations have been shown to be oncogenic through constitutive activation of the KRAS protein and the mitogen activated protein kinase (MAPK) pathway [7-12]. The universal observation of *KRAS* mutations in OSP suggests that these mutations represent an essential oncogenic event for these tumours.

Similar to OSP, 100% of SNSCC (including one adenosquamous carcinoma) arising in patients with concurrent or metachronous OSP (n = 5) harboured *KRAS* mutations (Figure 2). While OSP and ISP are occasionally observed in patients who present with SNSCC, the molecular

relationship between these papillomas and associated SNSCC is uncertain. In a previous study [3], we compared *EGFR* genotypes in matched pairs of ISP and associated SNSCC. In spite of the heterogeneity of *EGFR* mutations observed among tumours from different patients, identical *EGFR* genotypes were observed in matched ISP and associated SNSCC pairs. In the current study, material was available from 4 patients with OSP and concurrent associated SNSCC. Similar to our previous findings in ISP and associated SNSCC, separately extracted DNA from each matched OSP and associated SNSCC pair demonstrated identical *KRAS* genotypes (Figure 3). While the number of matched cases of OSP and associated SNSCC in this study is small, these results provide evidence to support the premise that sinonasal papillomas – including OSP – are precursor lesions for associated SNSCC. Indeed, although the mechanisms of malignant progression from sinonasal papilloma to SNSCC are not well-understood, high-risk human papillomavirus (HPV) infection is thought to play a role in a subset of ISP-associated SNSCC [13].

While *KRAS* mutations have been reported in a significant proportion of intestinal-type sinonasal adenocarcinoma [14-17], *KRAS* mutations are very uncommon in squamous cell carcinomas of the head and neck [18-20] and have only rarely been reported in SNSCC [14,21]. To determine the frequency of *KRAS* mutations in other similar sinonasal lesions, we evaluated a large cohort using Sanger sequencing (Figure 4; Supplementary Table 1). No *KRAS* mutations were identified in other sinonasal papillomas, including ISP (n = 50) and ESP (n = 5). In addition, *KRAS* mutations were not identified in ISP-associated SNSCC (n = 22) and were found in only 1 case

(5%) of SNSCC without a known papilloma association (n = 19). Between two previous studies, only one *KRAS* mutation (G12A) was detected among more than 100 unselected SNSCC [14,21]. It is uncertain if *KRAS* mutations truly do occur at a low frequency in *de novo* SNSCC or if the apparent absence of an associated OSP in these cases reflects insufficient clinical information, incomplete sampling, or overgrowth of an OSP by the associated SNSCC. Regardless, the universal presence of *KRAS* mutations in oncocytic sinonasal tumours (OSP and OSP-associated SNSCC), the absence of these mutations in other sinonasal papillomas, and the rarity of these mutations in other similar sinonasal lesions, however, suggests that *KRAS* mutations could be perceived as a disease-defining molecular feature of sinonasal oncocytic tumours.

The identification of *KRAS* mutations in OSP and OSP-associated SNSCC may also have important implications for therapy. In colorectal cancer, *KRAS* mutations are an important negative predictor of response to targeted anti-EGFR therapy [22]. Likewise, *KRAS* mutations in non-small cell lung cancer are mutually exclusive with *EGFR* mutations and portend a poor response to EGFR inhibitor therapy [23]. The absence of any benefit from EGFR-targeted therapy in tumours with *KRAS* mutations is thought to reflect the fact that KRAS is downstream of EGFR in the growth factor signalling pathway. Although not currently in wide use, a number of small molecule MEK inhibitors are in various phases of clinical trials and may someday be useful for the treatment of tumours with *RAS* mutations [24].

Collectively, the results of this study, our previously published data, and other previous studies provide a working model for oncogenic drivers in sinonasal papillomas: 1) OSP harbours *KRAS* mutations; 2) ISP contains *EGFR* mutations (and/or low-risk HPV infection); and, 3) ESP is associated with low-risk HPV infection [1,3,25]. The precise reason for this genotype-phenotype correlation is not readily apparent, particularly in light of the fact that activating mutations in *KRAS* and *EGFR* are likely to have similar functional consequences related to MAPK pathway activation. The phenotypic differences between ISP and OSP may reflect a difference in the cell of origin, associated secondary mutations, and/or activation of distinct gene expression programs. Alternatively (or in addition), oncocytic tumours are characterized by an aberrant number of mitochondria imparting a swollen appearance with abundant eosinophilic cytoplasm [26]. In many oncocytic tumours, the increase in mitochondria is linked to mitochondrial dysfunction through a variety of mechanisms [27]. Because *KRAS* mutations have been linked to mitochondrial dysfunction, these mutations may contribute to the oncocytic appearance of OSP [28]. Indeed, *KRAS* mutations in follicular thyroid lesions are associated with oncocytic change, in contrast to those with mutations in *NRAS* or *HRAS* – related genes in the growth factor signalling pathway [29].

Overall, our studies implicate a previously unknown and prominent role for activating *KRAS* mutations in the pathogenesis of oncocytic sinonasal tumours and suggest that *KRAS* mutations may be a disease-defining molecular feature of sinonasal oncocytic tumours. The presence of

KRAS mutations in these tumours indicates that EGFR targeted therapy is likely to be ineffective, while other treatments currently in clinical trials may one day play a therapeutic role.

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AUTHOR CONTRIBUTIONS

AMU, MSL, KSJEJ, NAB study design; AMU, JBM, KM, VAL, RRS, EY, OHI, BPO, KED, HCW data collection; AMU, JBM, NAB data analysis and data interpretation; AMU, NAB literature search; AMU, BLB, NAB generation of figures; AMU, BLB, MSL, KSJEJ writing of manuscript.

FIGURE LEGENDS

Figure 1. *KRAS* G12D mutation in OSP detected by targeted next-generation sequencing. (A) OSP initially evaluated by targeted next-generation sequencing. (B) Integrated Genomics Viewer representation of the *KRAS* G12D mutation detected by next-generation sequencing (shown in reverse complement). (C) Sequence electropherogram showing confirmation of *KRAS* G12D mutation by Sanger sequencing.

Figure 2. Frequencies of *KRAS* mutations in OSP and OSP-associated SNSCC.

Figure 3. Molecular relationship between OSP and associated SNSCC. (A) Histopathology and *KRAS* exon 2 sequence electropherograms for a separately extracted OSP (top) and concurrent associated SNSCC (bottom) from 1 patient demonstrating the same mutation (G12V). (B) *KRAS* genotypes for concurrent OSP and associated SNSCC from 4 unique patients.

Figure 4. *KRAS* and *EGFR* genotypes of sinonasal papillomas and squamous cell carcinoma. *KRAS* mutations are a universal finding in OSP and OSP-associated SNSCC but are not identified in ISP, ISP-associated SNSCC or ESP and are uncommon in SNSCC without a known papilloma association. In contrast, *EGFR* mutations are only detected in ISP and ISP-associated SNSCC [3].

SUPPORTING INFORMATION ON THE INTERNET

The following supporting information may be found in the online version of this article:

Table S1. *KRAS* and *EGFR* sequencing results in a large cohort of sinonasal papillomas and squamous cell carcinoma.

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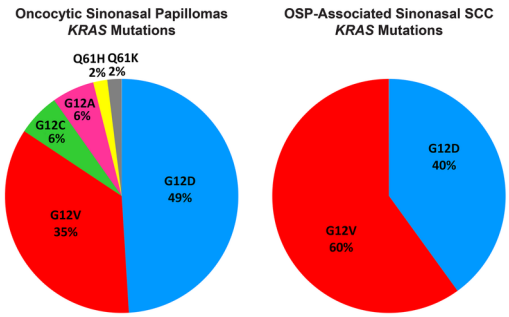

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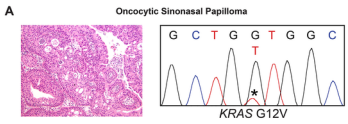

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	OSP *KRAS* Genotype		OSP-associated SNSCC *KRAS* Genotype	
Patient	cDNA	Protein	cDNA	Protein
1	35G>A	G12D	35G>A	G12D
2	35G>T	G12V	35G>T	G12V
3	35G>T	G12V	35G>T	G12V
4	35G>A	G12D	35G>A	G12D

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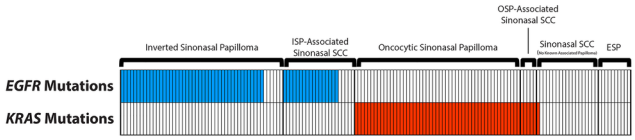

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