High-Frequency Targetable EGFR Mutations in Sinonasal Squamous Cell Carcinomas Arising from Inverted Sinonasal Papilloma

Aaron M. Udager¹, Delphine C.M. Rolland¹, Jonathan B. McHugh¹, Bryan L. Betz¹, Carlos Murga-Zamalloa¹, Thomas E. Carey², Lawrence J. Marentette², Mario A. Hermsen³, Kathleen E. DuRoss¹, Megan S. Lim¹, Kojo S.J. Elenitoba-Johnson¹, and Noah A. Brown¹

Abstract

Inverted sinonasal papilloma (ISP) is a locally aggressive neoplasm associated with sinonasal squamous cell carcinoma (SNSCC) in 10% to 25% of cases. To date, no recurrent mutations have been identified in ISP or SNSCC. Using targeted next-generation sequencing and Sanger sequencing, we identified activating EGFR mutations in 88% of ISP and 77% of ISP-associated SNSCC. Identical EGFR genotypes were found in matched pairs of ISP and associated SNSCC, providing the first genetic evidence of a biologic link between these tumors. EGFR mutations were not identified in exophytic or oncocytic papillomas or non–ISP-associated SNSCC, suggesting that the ISP/SNSCC spectrum is biologically distinct among sinonasal squamous tumors. Patients with ISP harboring EGFR mutations also exhibited an increased progression-free survival compared with those with wild-type EGFR. Finally, treatment of ISP-associated carcinoma cells with irreversible EGFR inhibitors resulted in inactivation of EGFR signaling and growth inhibition. These findings implicate a prominent role for activating EGFR mutations in the pathogenesis of ISP and associated SNSCC and rationalize consideration of irreversible EGFR inhibitors in the therapy of these tumors. Cancer Res; 75(13); 2600-6. ©2015 AACR.

Introduction

Inverted sinonasal papilloma (ISP) is a distinctive neoplasm arising from sinonasal (Schneiderian) epithelium. ISP usually arise from the lateral wall of the nasal cavity, from which they can extend along the sinonasal epithelium to involve the paranasal sinuses. Although they are considered benign, these tumors have unlimited potential for growth, local invasion, and destruction of contiguous structures that can lead to facial deformities and even death in some cases (1). Surgical resection is the treatment of choice, but recurrence is common (2). In addition, approximately 10% to 25% of ISP arise in association with a synchronous or metachronous sinonasal squamous cell carcinoma (SNSCC)—an aggressive, malignant disease with a 5-year mortality rate approaching 40% (3–5). Importantly, the genetic basis for ISP and associated SNSCC is currently unknown and, although ISP is presumed to be a precursor lesion for SNSCC, no previous study has established a molecular relationship between these tumors.

Materials and Methods

Specimens

With Institutional Review Board approval at the University of Michigan, we obtained archived formalin-fixed paraffin-embedded material from 50 patients with ISP (including 12 with matched SNSCC) and 22 patients with ISP-associated SNSCC (including 12 patients with matched papilloma material). ISP-associated SNSCC was defined as squamous cell carcinoma with either a concurrent or previously diagnosed conventional inverted papilloma. We also obtained specimens from 20 patients with SNSCC without clinical or pathologic evidence of an associated papilloma, 10 patients with exophytic sinonasal papillomas (ESP), and 5 patients with oncocytic sinonasal papillomas (OSP). All diagnoses were confirmed by an experienced head and neck pathologist (J.B. McHugh). For each case, DNA was separately extracted from areas of normal tissue and areas of papilloma and/or invasive squamous cell carcinoma using the Pinpoint Slide DNA Isolation System (Zymo Research) according to the manufacturer’s instructions. At least 30% tumor nuclei were required for papilloma and squamous cell carcinoma extractions. All areas of extraction were specified by an experienced head and neck pathologist (J.B. McHugh).

Cell lines

The UM-SCC-112 and UM-SCC-33 cell lines were obtained from the laboratory of Dr. Thomas E. Carey (University of Michigan, Ann Arbor, MI). UM-SCC-33 was derived from a
A 47-year-old female with SNSCC without any concurrent or previously diagnosed ISP (6). No EGFR mutations were identified in this cell line by Sanger sequencing. UM-SCC-112 has not been previously described in the literature but was derived from a 42-year-old female with ISP-associated SNSCC of the right nasal cavity and right maxillary sinus. The SCCNC4 cell line was obtained from the laboratory of Dr. Mario A. Hermsen (IUOPA, Asturias, Spain). This cell line was derived from a 73-year-old female with SNSCC of the right maxillary sinus who had previously been treated for an ISP involving the right nasal cavity (7). Sanger sequencing demonstrated an EGFR N771_H773dup mutation in UM-SCC-112 cells as well as in FFPE resection

Figure 1. Prevalence and distribution of EGFR mutations. A, prevalence of EGFR mutations in ISP (left) and ISP-associated SNSCC (right). B, distribution and frequency of EGFR exon 20 and exon19 mutations among ISP (n = 50; top) and ISP-associated SNSCC (n = 22; bottom).
material from which this cell line was derived. An **EGFR** S768_D770dup mutation was identified in SCCNC4 cells. HCC 827 cells were obtained from the ATCC and an **EGFR** E746_A750del mutation was confirmed by Sanger sequencing. UM-SCC-112, UM-SCC-33, and SCCNC4 cells were maintained in DMEM medium (Hyclone) supplemented with 10% heat-inactivated fetal bovine serum (HyClone), while HCC 827 cells were maintained in RPMI (HyClone) with 10% FBS. Cultures were kept at 37°C in a humidified atmosphere with 5% CO2.

**Ion AmpliSeq Cancer Hotspot Panel**

Targeted next-generation sequencing was performed on DNA from nine ISP, four SNSCC associated with inverted papillomas, and three SNSCC not associated with a papilloma. Sequencing libraries were generated using Life Technologies’ Ion AmpliSeq Cancer Hotspot Panel v2. Starting DNA (10 ng) from each sample block was amplified. Each library was barcoded (IonXpress Barcode Kit; Life Technologies) and equalized (Ion Library Equalizer Kit; Life Technologies) to a final concentration of approximately 100 pmol/L. Emulsification PCR using combined barcoded libraries was performed using the OneTouch 2 Instrument. Template-positive Ion Sphere particles were then enriched using the OneTouch ES Instrument per the manufacturer’s recommendations. Sequencing was performed on a 318v2 chip on the Ion Torrent PGM following the recommended protocol. Reads were aligned to hg19, and variants were called using the Torrent Suite 4.0.2 and Ion Reporter 4.0. Variants were assessed using the Broad Institute’s Integrated Genomics Viewer (IGV 2.3). Candidate somatic variants from the Ion AmpliSeq Cancer Hotspot Panel were defined as those in regions with a depth greater than 100× and a variant frequency greater than 10%. Synonymous variants and variants reported in 1000 Genomes were excluded.

**Sanger sequencing**

Based on next-generation sequencing results, all tumor specimens were evaluated using bidirectional Sanger sequencing with

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nested sequencing primers for EGFR exons 18 to 21. All observed mutations were confirmed to be somatic by sequencing of DNA extracted from matched normal tissue.

**Proliferation assay**

All cell lines were incubated in vehicle (DMSO), neratinib, afatinib, dacomitinib, gefitinib, or erlotinib at concentrations of 0.01 μmol/L, 0.1 μmol/L, 0.25 μmol/L, 0.5 μmol/L, 0.75 μmol/L, 1.0 μmol/L, 2.5 μmol/L, 5.0 μmol/L, 7.5 μmol/L, and 10.0 μmol/L. Cell growth was determined at 96 hours in triplicate by measurement of metabolic cleavage of tetrazolium salt into formazan using Cell Proliferation Reagent WST-1 (Roche Diagnostics) according to the manufacturer’s instructions. The IC50 value was determined from the regression of a plot of the logarithm of the concentration versus percent inhibition at the time point of maximal inhibition.

**Western blotting**

Cell lines were incubated in vehicle (DMSO), neratinib, or gefitinib at specified concentrations for 2 hours. Western blotting analyses were performed by the standard protocol using assorted primary antibodies: anti-pEGFR (pY1082, clone D7A5; Cell Signaling Technology), anti-EGFR (polyclonal; Cell Signaling Technology), anti-p-MEK 1/2 (pS217/221, polyclonal; Cell Signaling Technology), anti-MEK 1/2 (polyclonal; Cell Signaling Technology), anti-p-AKT (pS473, clone 587F11), and anti-AKT (polyclonal; Cell Signaling Technology).

**Statistical analysis**

Clinical variables, including age at diagnosis, gender, smoking history, ACC tumor (T) stage, margin status at primary resection, recurrence, progression to carcinoma, and/or death due to disease, were evaluated for potential association with EGFR mutation status. Continuous variables were evaluated using the Student t test, and categorical variables were assessed using the χ2 test. Recurrence-free survival (RFS), progression-free survival (PFS), and overall survival (OS) were calculated by the Kaplan–Meier method and compared using the log-rank test. For PFS, ISP without concurrent SNSCC were analyzed. Given the complete concordance observed between ISP and associated SNSCC EGFR genotypes, metachronous ISP-associated SNSCC without available ISP material were included in this analysis, and the EGFR genotype of the associated ISP was assumed to be the same as the sequenced SNSCC. Cox proportional hazards regression analysis was also used to evaluate the effect of EGFR mutation status on each outcome, both independently and after adjusting for specific clinical variables. Statistical analyses were performed using either the XLSTAT package (Addinsoft SARL) for Microsoft Excel or GraphPad Prism (version 5; GraphPad Software). A P value of <0.05 was considered statistically significant.

**Results and Discussion**

ISP is a distinctive, locally aggressive neoplasm frequently associated with SNSCC. Prior to this study, the pathogenesis and underlying genetic basis for ISP and SNSCC were unknown. To identify pathogenic somatic mutations in these tumors, we performed next-generation sequencing using a targeted mutation hotspot panel (Ion AmpliSeq Cancer Hotspot Panel) on fixed paraffin-embedded archival tissues comprising 9 ISP, 4 ISP-associated SNSCC, and 3 non–ISP-associated SNSCC. EGFR mutations were identified in 7 of 9 ISP and 3 of 4 ISP-associated SNSCC. All mutations were confirmed to be somatic by Sanger sequencing of DNA from both tumor and matched normal tissue. No mutations were observed in any of the 3 non–ISP-associated SNSCC.

EGFR mutations are extremely rare in squamous cell carcinomas of the head and neck as a whole (8) as well as in sinonasal adenocarcinoma (9, 10). However, no study has specifically evaluated SNSCC or ISP for EGFR mutations. We explored the frequency and spectrum of EGFR mutations in ISP and associated SNSCC by evaluating a larger cohort of cases using Sanger sequencing of EGFR exons 18 to 21. Sequencing a total of 50 ISP and 22 ISP-associated SNSCC demonstrated EGFR mutations in 88% and 77% of tumors, respectively (Fig. 1A). A total of 19 different EGFR mutations were identified (Fig. 1B). Only one mutation was identified in each tumor, and each mutation was shown to be somatic by sequencing of DNA extracted from matched normal tissue. A similar distribution of mutations was observed in ISP and ISP-associated SNSCC. Interestingly, the majority of EGFR mutations were exon 20 insertions (96% of ISP and 88% of ISP-associated SNSCC), with the remainder comprised of exon 19 deletions and nucleotide substitutions. This is in contrast with lung adenocarcinoma, in which EGFR exon 19 deletions predominate and exon 20 insertions account for 4% to 9% (11). Although a variety of EGFR exon 20 insertions were observed in ISP and ISP-associated SNSCC, all mutations involved residues located between A767 and C775. These mutations affect the loop following the C-helix region of the kinase domain and have been shown to result in constitutive activation of EGFR in lung adenocarcinoma (10).

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Although ISP has long been assumed to be a precursor for SNSCC, this relationship has never been proven at the molecular level, and recent X chromosome inactivation studies have called this assumption into question (12). To characterize the molecular relationship of ISP and SNSCC, we evaluated material from 12 patients with an ISP and either a synchronous (8) or metachronous (4) SNSCC. For each patient, DNA was separately extracted from each tumor (i.e., ISP and associated SNSCC) and subjected to EGFR sequencing (Fig. 2A). Although a variety of different mutations were identified in this study, identical EGFR genotypes were observed in each ISP/SNSCC pair (Fig. 2B). This relationship was observed in both synchronous and metachronous cases. These findings provide the first molecular evidence to support the role of ISP as a precursor for SNSCC and suggest that EGFR mutations are early events in the pathogenesis of ISP-associated SNSCC.

In addition to ISP and associated SNSCC, other squamous lesions involving the nasal cavity and sinuses include ESP, OSP, and SNSCC without an associated ISP (1). All of these lesions derive from sinonasal (Schneiderian) epithelium. ESP differ from ISP in both their growth pattern and their location, occurring more frequently on the nasal septum rather than on the lateral nasal wall. Malignant transformation is also extremely unusual in ESP (1). Several studies have implicated human papillomavirus (HPV) in the genesis of ESP (13). Although HPV DNA has also been detected in ISP, this may reflect incidental colonization rather than a pathogenic event (14). In contrast, the high-frequency, activating EGFR mutations observed in this study are likely to be significant, initiating events in ISP pathogenesis. Similar to ISP, OSP arises from the lateral nasal wall or paranasal sinuses and is associated with SNSCC in 4% to 17% of cases (1). HPV is not thought to play a role in OSP. Despite significant clinicopathologic overlap, OSP have a unique histologic appearance that allows them to be distinguished from ISP by routine pathologic examination. OSP tumor cells classically have abundant oncocytic cytoplasm by light microscopy and numerous mitochondria ultrastructurally (15). To determine the specificity of EGFR mutations among squamous sinonasal lesions, we evaluated a total of 20 non–ISP-associated SNSCCs, 10 ESP, and 5 OSP using Sanger sequencing of EGFR exons 18 to 21 (Fig. 3A).

No EGFR mutations were identified in any of the non–ISP-associated SNSCC, ESP, or OSP. The high frequency and...
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specificity of EGFR mutations observed in this study suggests that the ISP/SNSCC disease spectrum is biologically distinct from these other sinonasal squamous lesions.

ISP is associated with SNSCC in 10% to 25% of cases. Approximately 40% of associated carcinomas are metachronous and develop, on average, 63 months (or up to 13 years) after the diagnosis of ISP (4). Predicting which ISP patients will progress to SNSCC is difficult as there is no correlation between clinical variables, such as the number of ISP local recurrences, and the subsequent development of carcinoma. Clinical and genotypic information for our cohort of sinonasal papilloma and SNSCC is shown in Supplementary Tables S1 and S2, respectively. Importantly, EGFR mutation status in ISP demonstrated prognostic significance with EGFR wild-type ISP being associated with earlier progression to SNSCC (log-rank < 0.01; Fig. 3B). This association was also significant in both univariate and multivariate Cox regression analyses (Supplementary Tables S3 and S4). This finding implicates EGFR mutation status as a potential prognostic marker that may be useful in predicting which ISP patients will progress to SNSCC. No other statistically significant clinical association with EGFR status was identified in either ISP or ISP-associated SNSCC (Supplementary Tables S5 and S6).

Several small-molecule inhibitors targeting EGFR are FDA approved or in clinical trials for the treatment of neoplasms with activating EGFR mutations—principally EGFR-mutated lung cancer. In contrast with lung cancers with EGFR ex19 deletions, those with exon 20 insertions are generally resistant to the currently available reversible EGFR inhibitors gefitinib and erlotinib (16). However, several studies have shown more robust in vitro sensitivity of exon 20–mutated lung cancers to irreversible EGFR inhibitors (17–20), and the therapeutic potential of irreversible inhibitors is currently being investigated in clinical trials. In order to evaluate the functional significance of EGFR mutations in ISP/SNSCC and to assess the potential utility of therapeutic agents targeting these mutations, we obtained two cell lines derived from ISP-associated SNSCC—SCCNC4 and UM-SCC-112. EGFR mutations were found in both cell lines (EGFR S768_D770dup and N771_H773dup, respectively). These cell lines were compared with UM-SCC-33 (a non–ISP-associated SNSCC cell line found to be EGFR wild-type) and HCC 827 (an inhibitor-sensitive lung cancer cell line with an EGFR exon 19 deletion; E746_A750del). Cell lines were treated with two reversible EGFR inhibitors (erlotinib and gefitinib) and three irreversible EGFR inhibitors (neratinib, afatinib, and dacomitinib). As expected, both ISP-associated SNSCC cell lines (SCCNC4 and UM-SCC-112) were relatively resistant to reversible EGFR inhibitors with IC_{50} values from 913 nmol/L to >1,000 nmol/L (Fig. 4A and B). However, irreversible inhibitors, particularly neratinib, showed much more potent growth inhibition with IC_{50} Value as low as 143 nmol/L. By comparison, the lung cancer cell line with an EGFR exon 19 deletion (HCC 827) was sensitive to both classes of inhibitors (IC_{50} < 169 nmol/L), and EGFR wild-type, non–ISP-associated SNSCC (UM-SCC-33) was resistant to all inhibitors (IC_{50} > 10,000 nmol/L). Furthermore, treatment of ISP-associated SNSCC cells (SCCNC4) with the irreversible inhibitor neratinib strongly inhibited activation of EGFR and its downstream signaling mediators MEK and AKT, while such abrogation was only seen with high doses of the reversible inhibitor gefitinib (Fig. 2C). In contrast, HCC 827 cells showed robust abrogation of EGFR/MEK/AKT signaling with both types of inhibitors. It is unclear if the concentrations of irreversible EGFR inhibitors required to cause in vitro growth inhibition of ISP-associated SNSCC are clinically achievable in patients. However, these findings identify irreversible EGFR inhibitors as the first potential means of targeted therapy in ISP-associated SNSCC.

Overall, our studies implicate a previously unappreciated and prominent role for activating EGFR mutations in the pathogenesis of ISP and ISP-associated SNSCC. In addition, we provide the first genetic evidence that these neoplasms represent entities within the same spectrum of tumor evolution. Finally, our studies rationalize consideration of irreversible EGFR inhibitors in the therapy of ISP and ISP-associated SNSCC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: A.M. Udager, D.C.M. Rolland, M.S. Lim, K.S.J. Elenitoba-Johnson, N.A. Brown
Development of methodology: D.C.M. Rolland, B.L. Betz, K.S.J. Elenitoba-Johnson, N.A. Brown
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.M. Udager, D.C.M. Rolland, J.B. McHugh, C. Murga-Zamalloa, K.E. DuRoss, N.A. Brown
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.M. Udager, D.C.M. Rolland, J.B. McHugh, B.L. Betz, T.E. Carey, M.S. Lim, N.A. Brown
Writing, review, and/or revision of the manuscript: A.M. Udager, J.B. McHugh, C. Murga-Zamalloa, T.E. Carey, L.J. Marentette, M.A. Hermens, M.S. Lim, K.S.J. Elenitoba-Johnson, N.A. Brown
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.M. Udager, L.J. Marentette, M.A. Hermens, K.E. DuRoss
Study supervision: M.S. Lim, K.S.J. Elenitoba-Johnson, N.A. Brown

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papilloma: report of 6 cases with systematic review and pooled analysis.
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