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IDENTIFICATION OF NOVEL FIBROBLAST GROWTH FACTOR RECEPTOR 3 GENE MUTATIONS IN ACTINIC CHEILITIS

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Abstract

Objective—Activating mutations in the fibroblast growth factor receptor 3 (FGFR3) gene are responsible for several craniosynostosis and chondrodysplasia syndromes as well as some human cancers including bladder and cervical carcinoma. Despite a high frequency in some benign skin disorders, FGFR3 mutations have not been reported in cutaneous malignancies. Actinic cheilitis (AC) is a sun-induced premalignancy affecting the lower lip that frequently progresses to squamous cell carcinoma (SCC). The objective of this study was to determine if FGFR3 gene mutations are present in AC and SCC of the lip.

Study Design—DNA was extracted and purified from micro-dissected, formalin-fixed, paraffin-embedded tissue sections of 20 cases of AC and SCC arising in AC. Exons 7, 15, and 17 were PCR amplified and direct sequenced.

Results—Four novel somatic mutations in the FGFR3 gene were identified: exon 7 mutation 742C→T (amino acid change R248C), exon 15 mutations 1850A→G (D617G) and 1888G→A (V630M), and exon 17 mutation 2056G→A (E686K). Grade of dysplasia did not correlate with presence of mutations.

Conclusion—The frequency of FGFR3 receptor mutations suggests a functional role for the FGFR3 receptor in the development of epithelial disorders and perhaps a change may contribute to the pathogenesis of some AC and SCC.

INTRODUCTION

Fibroblast growth factor receptor 3 (FGFR3) belongs to a family of high affinity transmembrane tyrosine kinase receptors that modulate diverse biological processes during embryogenesis and tissue homeostasis.^{1–3} These cell surface glycoproteins are composed of an extracellular ligand-binding domain containing two to three immunoglobulin Ig-like domains, a transmembrane domain, and an intracellular split tyrosine kinase domain.⁴ Extracellular binding of fibroblast growth factors (FGFs) in the presence of heparin sulfate proteoglycan induces FGFR dimerization resulting in consecutive transphosphorylation of intracellular tyrosines and downstream phosphorylation of signaling proteins.^{3, 5–7} Alternate

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mRNA splicing within the juxtamembrane Ig-like domain generates two variant FGFR3 isoforms, differing in ligand-binding specificities and tissue distribution.⁸ The FGFR3b is predominantly expressed in epithelial cells whereas FGFR3c is found primarily in chondrocytes.^{1, 3, 9, 10}

Specific germline point mutations affecting differing domains of the FGFR3 gene are associated with several autosomal dominant skeletal dysplasias: dwarfism and several craniosynostosis syndromes including hypochondroplasia, severe achondroplasia with developmental delay and acanthosis nigricans (SADDAN), thanatophoric dysplasia, Crouzon syndrome with acanthosis nigricans, and coronal craniosynostosis.^{11–14} The same mutations that are seen in these skeletal dysplasias have also been identified in several human cancers and thus it has also been suggested that FGFR3 mutations may be oncogenic³. For example, activating FGFR3 somatic mutations have been reported in 25% of cervical carcinoma and 35% of urothelial carcinoma suggesting that the constitutive activation of FGFR3 serves as an important mechanism underlying the pathogenesis of these epithelial malignancies.^{15, 16}

Functional studies have shown that FGFR3 mutations generate a constitutively active tyrosine kinase through differing mechanisms depending on the type and position of amino acid substitution.^{3, 17} For example, those mutations that generate a cysteine residue, such as the R249C mutation commonly found in urothelial and cervical cancer,¹⁵ induce ligand-independent stabilization and auto-phosphorylation of the receptor through inter- and intra-molecular disulfide bond formation.^{18, 19}

Recently, a high frequency of the FGFR3 mutations have been identified in benign cutaneous disorders including 26.1% of epidermal nevi and 85% of adenoid seborrhic keratosis.^{20, 21} These findings suggest that the FGFR3 gene may play a role in cutaneous biology and that mutations of the FGFR3 gene may contribute to the development of some epithelial neoplasms. However, to date, FGFR3 mutations have not been identified in cutaneous malignancies. Actinic cheilitis (AC) is a precancerous lesion affecting the lower lip that frequently develops into squamous cell carcinoma (SCC). In this study, we investigated the presence of FGFR3 mutations in AC and SCC arising in AC.

MATERIALS AND METHODS

Tissue Specimens & Clinical Data

Formalin-fixed, paraffin embedded tissue biopsies of AC (n=15) and SCC arising in AC (n=5) cases were retrieved from the University of California, San Francisco Oral Cancer Research Center. All cases were reviewed to confirm the diagnosis and assessed for suitability for DNA analysis. Clinical data including patient demographics was retrieved from the OCRC database. The collection and utilization of all clinical and demographic data was approved by the University of California San Francisco's Institutional Review Board.

Microdissection and DNA Extraction

Microdissection was performed on five 10µm sections of formalin-fixed, paraffin embedded tissue sections to isolate lesional and non-neoplastic tissues. A single section was stained and examined at the end of tissue processing to ensure that lesional tissue remained. Using hematoxylin and eosin-stained sections, lesional tissues were identified and mapped under direct microscopic visualization and then separated from unstained sections using new scalpel blades. DNA was extracted using the QIAamp DNA Micro Kit (Qiagen, Valencia, CA, USA) by the protocol supplied by the manufacturer with two additional overnight incubations supplemented with proteinase K at 70°C.

PCR Amplification

PCR primers were designed to amplify exons 7, 15, and 17 of the FGFR3 gene, regions known to harbor point mutations in other cancers. Primers were as follows: exon 7: 5'-GAGAACAAAGTTTGGCAGCATC-3' and 5'-AACCCCTAGACCCAAATCCTC-3'; exon 15: 5'-GAGTACTTGGCCTCCAGAA-3' and 5'-GGTGAGTGTAGACTCGGTCAAA-3'; and exon 17: 5'-GTGTGGTTTCTACCCCTCCC-3' and 5'-TATTCGGAACAGCCTGAAG-3'. Primer sequences were created using the UCSC Genome Bioinformatics Database (<http://www.genome.ucsc.edu>). Real-time PCR was performed using Power SYBR Green master mix (Applied Biosystems, Forest City, CA, USA). PCR was carried out with the following conditions in a 7900HT instrument (Applied Biosystems, Forest City, CA, USA): 10 µl total reaction volume, 200nM FW/R primers, 2x Power SYBR Green master mix. 1 ng of genomic DNA was used per reaction. Cycling conditions were as follows: 95°C for 10 minutes, 50 cycles at 95°C for 15 seconds, 60°C for 1 minute, followed by 1 cycle at 95°C for 15 seconds, 60°C for 15 seconds, 95°C for 15 seconds. Negative (no DNA) and positive controls (human male DNA) were included in each RT-PCR reaction. A dissociation curve was assessed to determine FGFR3 exon product purity. PCR products were visualized after electrophoresis in 2% agarose to isolate and confirm fragments of expected size.

DNA Sequencing

Using the QIAquick Gel Extraction Kit (Qiagen Corp., Valencia, CA, USA), PCR products were isolated and then direct sequenced using ABI BigDye Terminator v3.1 (Applied Biosystems, Forest City, CA, USA) on an ABI 3730x1 DNA PCR Analyzer. Sequencing was performed in the 5' and 3' directions using the same PCR primers. DNA sequence analysis was performed using Sequencer (Gene Codes, Ann Arbor, MI, USA) and UCSC Genome Bioinformatics Database (<http://www.genome.ucsc.edu>).

FGFR3 Immunohistochemistry

Formalin-fixed, paraffin embedded tissues were stained by immunohistochemistry using a polyclonal antibody raised against the cytoplasmic region of human FGFR3 (Santa Cruz, CA) diluted 1:500 and pre-treated with trypsin digest. Sections were deparaffinized in xylene followed by rehydration in graded ethanol and then endogenous peroxidase activity was blocked using 3% hydrogen peroxide and non-specific antibody binding blocked with 2% BSA solution for 20 min. Application of secondary antibodies was performed for 1 hour at room temperature followed by application of Vectastain® Elite ABC (Vector Labs, Burlingame, CA). The bound antibody complexes were visualized by application of AEC substrate (DAKO, Carpinteria, CA).

Results

Clinical Results

Of the twenty cases selected for study, eight (40.0%) were obtained from women and twelve (60.0%) from men. All cases occurred on the lower lip. The ages of the patients ranged from 39 to 88 with a mean age of 55.3 years. Among the cases of AC, histological subtypes included AC without dysplasia (n=4), AC with mild dysplasia (n=4), AC with moderate dysplasia (n=5), AC with severe dysplasia (n=2). There were 5 cases of SCC arising from pre-existing AC (n=5).

Four Novel Somatic FGFR3 mutations found in AC and SCC

Exons 7, 15, and 17 of the FGFR3 gene were successfully amplified from all AC and SCC cases. Mutations were identified in 4 of 20 (20%) cases (Tables 1 & 2). A mutation in exon 7,

corresponding to the linker region between immunoglobulin Ig-like domain II-III, was found in a case of AC with severe dysplasia. This C-to-T substitution at nucleotide position 742 resulted in a predicted amino acid change of arginine to cysteine at position 248 (R248C). Two mutations in exon 15, corresponding to the tyrosine kinase domain II, were found in an SCC and in a case of AC with mild dysplasia. The SCC case had a substitution of an A-to-G at nucleotide 1850 resulting in a predicted amino acid change of aspartic acid to glycine at position 617 (D617G). The AC with mild dysplasia contained a G-to-A substitution at nucleotide 1888, causing a change from valine to methionine at amino acid position 630 (V630M). Finally, a mutation in exon 17, corresponding to the tyrosine kinase domain II was found in one case of AC without dysplasia. This G-to-A substitution at nucleotide 2056, resulted in a predicted amino acid change of glutamic acid to lysine at position 686 (E686K) (Figure 1). In cases with mutations in lesional tissue the DNA sequences from corresponding non-neoplastic tissue was sequenced and shown to contain wild-type FGFR3 sequence confirming somatic and not germline mutations.

FGFR3 Mutational Status is Not Related To Histologic Grade

FGFR3 mutations were found in all histological grades of AC dysplasia: AC without dysplasia (E686K), AC mild dysplasia (V630M), AC severe dysplasia (R248C), and in SCC (D617C). There was no association between FGFR3 mutational status and histological grade of AC or SCC (Table 2).

Single Nucleotide Polymorphisms Identified in AC and SCC

We detected 6 different single nucleotide polymorphisms arising in 7 of 20 (35%) cases. None resulted in amino acid changes. A case of AC with mild dysplasia and SCC contained a C-to-T substitution at nucleotide 753 at amino acid position 251; two SCC cases and an AC with moderate dysplasia contained a T-to-C substitution at nucleotide 882 at amino acid position 294; a case of AC with mild dysplasia showed a C-to-T substitution at nucleotide 1839 at amino acid position 613; a case of AC with moderate dysplasia showed a C-to-T substitution at nucleotide 1941 at amino acid position 647; SCC had a C-to-T substitution at nucleotide 2148 at amino acid position 716. There was no association between presence of silent point mutations and grade of AC dysplasia or SCC (Table 1).

Immunohistochemistry

FGFR3 protein expression was identified by immunohistochemistry in all mutant and non-mutated cases of AC and SCC. In dysplastic epithelium the pattern of expression was strongest in the prickly layer as previously reported (Figure 2).²² There was diffuse expression in tumor cells of SCC.

DISCUSSION

This study has identified somatic mutations of the FGFR3 gene in a large proportion of AC and SCC of the lip. AC is a premalignant condition of the lip associated with excess exposure to ultraviolet light. The condition most commonly occurs on the lower lip of fair-skinned persons and, if left untreated, has a significant risk of transformation to SCC. The histological appearance of the epithelium in AC may vary from thinning to varying grades of dysplasia ranging from mild to severe. SCC is characterized by invasion of malignant epithelium into the underlying connective tissues.²³ Because of its occurrence on the vermilion border of the lip and its association with excess sun-exposure, the biology of AC is more similar to other cutaneous premalignant conditions such as actinic keratosis than oral cavity premalignancy.²⁴ Treatment of AC depends on the severity with mild cases requiring the application of sunscreens to surgery for more severe cases and for SCC.²⁵

Recently somatic mutations of the FGFR3 gene have been identified in bladder and cervical carcinomas and in a subset of multiple myeloma suggesting that this change plays a role in some human cancers. To date, mutations of the FGFR3 gene have not been reported in squamous cell carcinomas of the skin or mucosa of the head and neck. On the skin, FGFR3 mutations were first identified in some benign epithelial disorders such as epidermal nevi and adenoid seborrhic keratoses. Logie et al. (2005) showed that transgenic mice with a S249C mutation in the FGFR3 protein developed benign epithelial tumors but with no malignant potential.¹⁷ Here, we show that FGFR3 mutations are found in 20% (4/20) of the cases of AC and SCC of the lip. The four somatic mutations identified in AC and SCC were identified in exons 7, 15, and 17, regions known to harbor most of the FGFR3 mutations previously described in other malignancies. While FGFR3 mutations may not be limited to these exons, other studies have examined other exonic regions in SCC of the skin and mouth but failed to identify mutations.¹⁴ In addition to targets for mutation in other cancers, exons 7, 15 and 17 were selected for analysis in this study because they code for proteins involved in the structure and downstream signaling of the tyrosine kinase receptor. Exon 7 encodes for the linker region between Ig-like immunoglobulin domain II–III in the extracellular ligand-binding domain whereas exon 15 and 17 are involved in the synthesis of the intracellular tyrosine kinase domain II.

While the effects of somatic FGFR3 mutations have been not fully elucidated, some studies have shown that mutations affecting these regions produce conformational protein changes that generate ligand-independent binding sites resulting in a constitutively active FGFR3 protein. For instance, the R248C mutation located in exon 7 present in an AC with severe dysplasia represents one of the most frequent genetic alterations described in some benign epithelial disorders.^{17, 20} The C-to-T transition results in an amino acid change of arginine to cysteine inducing dimerization of the tyrosine kinase receptor through intermolecular disulfide bond formation.^{3, 18} The mutations in exons 15 and 17 identified in this study disrupt the tyrosine kinase domain II but have, to date, not been reported in a cutaneous disorder. However, other mutations affecting the TK II domain have been well described, and functional studies show that genetic alternation to this region stabilizes the active conformation of the FGFR3 receptor with little ligand dependence.²⁶

Mutations of the FGFR3 gene were seen in both early and late stage AC and in SCC. Of the 4 mutations identified, two mutations occurred in the earliest stages of the disease in an AC without dysplasia and in an AC with mild dysplasia. Since this was a cross-sectional study that did not follow individual lesions for disease progression over time it is difficult, with certainty, to determine if the two remaining mutations of the FGFR3 gene that were found in AC with severe dysplasia and SCC were also present at an earlier stage in development. It is conceivable that FGFR3 mutations may be an early event in their development but this would need to be further evaluated in longitudinal studies. The importance of this issue is that the identification of early mutagenic events would be relevant clinically for screening, early intervention and possible targeted therapies.

Other molecular events have been reported in the development of lip SCC including overexpression of the p53 protein, mutations of the p53 gene particularly C-toT transitions at dipyrimidine sites typical of UV radiation exposure²⁴ and reduced expression of bcl-2 protein.²⁷ In other tumor sites, FGFR3 mutations are often associated with upregulation of the p53 protein such as in urothelial carcinoma²⁸ and bcl-2 protein in seborrhic keratosis.²⁹ The relationship between these molecular changes and their biological significance in lip cancer however is not yet known.

As a treatment site, the lip offers many advantages including ease of disease identification, therapeutic monitoring and follow-up. However, current therapies for both late stage AC and

SCC of the lip are surgical that result in some treatment associated morbidity.²⁵ Mutations of the FGFR3 tyrosine kinase gene in several benign and malignant disorders including lip SCC raises the possibility of targeted therapies for the disease. For example, the small molecular tyrosine kinase inhibitors PD173074, SU5402, and PKC412³⁰ are available and are currently in phase II trials.³¹ The PKC412 inhibitor has already shown success for the treatment of FGFR3-induced hematopoietic malignancy.³² The identification of FGFR3 mutations in a large proportion of lip AC and SCC is the first necessary step to begin to identify alternative, targeted therapies for this condition.

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Figure 1.

A: Case 3 (AC). Top: G-to-A substitution at nucleotide 2056 (exon 17) causing a predicted amino acid change from glutamic acid to lysine at codon 686 of the TKII domain. Lower: wild-type DNA sequence in the adjacent non-neoplastic tissue.

B: Case 8 (AC Mild Dysplasia). Top: G-to-A substitution at nucleotide 1888 (exon 15) causing a predicted amino acid change from valine to methionine at codon 630 of the TKII domain. Lower: DNA in the adjacent non-neoplastic tissue was wild-type.

C: Case 15 (AC Severe Dysplasia). Top: C-to-T substitution at nucleotide 742 (exon 7) causing a predicted amino acid change from arginine to cysteine at codon 248 in the linker region between immunoglobulin Ig-like domain II–III. Lower: wild-type DNA in the adjacent non-neoplastic tissue.

D: Case 17 (SCC). Top: A-to-G at nucleotide 1850 (exon 15) causing a predicted amino acid change from aspartic acid to glycine at position 617 in the TKII domain. Lower: wild-type DNA in adjacent non-neoplastic tissue.

Figure 2.

A: Immunohistochemical demonstration of FGFR3 protein expression in AC with wild-type FGFR3 gene (40x). B: Immunohistochemical demonstration of FGFR3 protein expression in an SCC arising from AC with a point mutation in exon 15 of the FGFR3 gene (40x).

Table 1
Clinical data and FGFR3 mutational status in AC and SCC

Case	Age	Gender	Diagnosis	Exon	Mutation	Predicted AA change & position	FGFR3 protein
1	54	F	AC				+
2	44	M	AC				+
3	39	F	AC	17	2056G→A*	E686K	+
4	51	F	AC				+
5	62	M	AC Mild Dysplasia	7 15	753C→T 1839C→T	251 (Si) 613 (Si)	+
6	57	M	AC Mild Dysplasia				+
7	44	M	AC Mild Dysplasia				+
8	63	M	AC Mild Dysplasia	15	1888G→A*	V630M	+
9	85	M	AC Moderate Dysplasia				+
10	47	M	AC Moderate Dysplasia				+
11	53	M	AC Moderate Dysplasia				+
12	60	F	AC Moderate Dysplasia	15	1941C→T	647 (Si)	+
13	49	F	AC Moderate Dysplasia	7	882T→C	294 (Si)	+
14	39	M	AC Severe Dysplasia				+
15	53	F	AC Severe Dysplasia	7	742C→T*	R248C	+
16	88	F	SCC	17	2148C→T	716 (Si)	+
17	70	M	SCC	15	1850A→G*	D617G	+
18	58	M	SCC	7	882T→C	294 (Si)	+
19	41	M	SCC	7	882T→C	294 (Si)	+
20	49	F	SCC	7	753C→T	251 (Si)	+

F = female; M = male; AC = actinic cheilitis; SCC = squamous cell carcinoma;

* DNA sequence of microdissected normal tissue was wild-type confirming somatic and not germline mutation; (Si) = silent mutation; E= Glutamic acid; K = Lysine; V= Valine; M= methionine; R= arginine; C= cysteine; D= Aspartic acid; G= Glycine

Table 2

Summary of somatic FGFR3 mutations in AC and SCC, corresponding amino acid change, and functional effect

Diagnosis	FGFR3 protein status	Functional domain location	Functional effect of AA change
AC	E686K	TKII; conserved	polar→polar
AC with mild dysplasia	V630M	TKII; conserved	nonpolar→nonpolar
AC with severe dysplasia	R248C	Linker Ig II–III; conserved	polar→nonpolar
SCC	D617G	TKII; conserved	polar→nonpolar

TK = tyrosine kinase; Ig = immunoglobulin domain