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Targeted Sequencing of an Epidemiologically Low Risk patient defines Fibroblast Growth Factor Family Aberrations as a putative driver of Head and Neck Squamous Cell Carcinoma

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Abstract

Background—Targeted sequencing of epidemiologically low risk (ELR) HNSCC patients could help identify novel drivers or lost suppressors leading to precision medicine protocols and improved survival rates.

Methods—An ELR-HNSCC patient was selected for targeted sequencing. We then assessed next generation sequencing cohorts from the Oncomine Powertool Database, which contains pan-cancer data from The Cancer Genome Atlas(TCGA).

Results—Targeted sequencing revealed *FGFR1* amplifications as a putative driver of the patient's tumor. HNSCC patients from TCGA data demonstrated *FGF* family mutations, rearrangements or amplifications in over 35% of HNSCC cases, with a statistically significant higher frequency in African American populations. *FGF* alterations were unique from activating *PIK3CA* mutations.

Conclusion—Together, this data suggests that *FGF* signaling may be critical for a subset of HNSCC patients independent of other known pathways and provides rationale for leveraging ELR-HNSCC patients to define molecular subsets of high risk HNSCC.

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The authors of this study report no commercial association or competing financial interests.

Keywords

HNSCC; FGF; FGFR; Amplification; Mutant

INTRODUCTION

There are approximately 560,000 new cases of head and neck squamous cell carcinoma (HNSCC) resulting in 300,000 deaths per year worldwide.[1] For these patients, prognosis is often poor due to disease propensity for extensive local invasion, early dissemination into regional lymph nodes and metastatic spread.[2] Despite modifications in treatment over the past decade, overall survival has remained unchanged for advanced disease with a less than a 50% five-year survival rate.[3]

To improve patient outcomes in HNSCC, a change in our treatment approach may be necessary. Cancer genomics is a promising field that seeks further understanding of the genetic drivers of these tumors. These drivers may in turn allow for the discovery of druggable targets. While these types of precision medicine protocols are currently in phase II and III trials for multiple cancer types, no protocols exist for HNSCC due to a lack of comprehensive knowledge of disease genetics and molecular targets.[4,5]

Several groups, including The Cancer Genome Atlas (TCGA), are currently using next generation sequencing in order to molecularly subtype HNSCC.[6] While sequencing studies completed thus far have confirmed that there are multiple molecular alterations in HNSCC, the observed distribution of mutations from the high risk patients is incredibly complex, especially compared with other tumors that are not related to smoking and alcohol use. On average, 141 mutations per tumor are found in the HNSCC TCGA data, with no clear distinction of driver and passenger mutations.[6] Attempts to molecularly stratify are further confounded by the fact that many of these mutations occur in different genes within an individual pathway (functional recurrence).

As it is well established that tobacco and alcohol use lead to increased mutational load, there is the potential for increased prevalence of passenger mutations, making identifying the underlying driver mutations more difficult to identify. Additionally, Human Papilloma Virus (HPV) is a known cause of HNSCC.[3] The mutational load in HPV-negative patients who do not smoke or drink, or epidemiologically low risk (ELR) patients, potentially may be lower, and without established genetic drivers such as HPV E6/E7. As such, mutations identified in these patients have a greater chance of being clinically relevant. In light of this fact, we began our analysis by examining the disruptive genomic events in an ELR patient with aggressive recurrent HNSCC. The purpose of this study is to profile a HNSCC tumor within a low risk patient to understand the possible genetic drivers, and then secondarily to analyze these putative genetic drivers within a larger genetically sequenced cohort.

MATERIALS AND METHODS

Patient Material

This study carries University of Michigan Institutional Review Board approval under HUM00080561. An ELR HNSCC patient (UM-ELR -01) was selected for targeted sequencing on DNA isolated from a diagnostic formalin-fixed, paraffin-embedded (FFPE) tissue block. Representative FFPE sections with >50% tumor content were used for molecular analysis. Sections were macrodissected to enrich for tumor content and cut for DNA extraction using the Qiagen Allprep FFPE DNA kit according to manufacturer's instructions. DNA was then quantified using the Qubit fluorometer as described.[7]

Targeted DNA sequencing

Amplicon based DNA sequencing and data analysis was performed using 40 ng of isolated DNA on the Ion Torrent Personal Genome Machine (PGM) utilizing the AmpliSeq Comprehensive Cancer Panel as described.[7] Nucleotide variants and indels were identified using the Torrent Variant Caller plugin, annotated using Annovar[8], and filtered to include candidate somatic mutations by removing germ line variants and sequencing artifacts using in-house validated pipelines.[7,9] Copy number alterations were identified as described[7,10,11], using normalized, GC content corrected, total read counts per amplicon from UM-ELR-01 divided by those from a composite "normal" sample consisting of over 600 DNA samples. Gene-level copy number estimates were determined by taking the coverage-weighted mean of the per-probe ratios, with expected error determined by the probe-to-probe variance. We sequenced patient UM-ELR-01 using a single 318 chip to generate 3,819,576 mapped reads, 98.54% of which were on target. The 1,688,650 targeted bases were covered to an average depth of 235×, with 95.12% of targeted bases covered at >20×. Variants were filtered through a standardized pipeline to remove low confidence calls, sequencing errors, germline variants and common polymorphisms to prioritize potential driving non-synonymous, somatic alterations.

Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) was performed from FFPE tissue slides from both the primary and the recurrent tumor of UM-ELR-01 to confirm DNA amplification. This was performed using standard protocols. Briefly, probes were used against FGFR1 and Centromere 8 (Cat. #FGFR1-20-OR, Empire Genomics, Buffalo, NY). They were subsequently blocked with 10% goat serum (Sigma-Aldrich, St. Louis, MO) plus 90% CAS block (Invitrogen, Carlsbad, CA). Slides were blotted with 1:500 anti-digoxigenin-fluorescein (Roche, South San Francisco, CA) and streptavidin-alexa flour 594 (Invitrogen) was placed for 1 hour. DAPI (Invitrogen) was applied to the slides before visualizing under fluorescent microscopy.

Western Blot

Protein isolation and western blot was performed from the recurrence of patient UM-ELR-01 tissue block to confirm protein expression (there was not enough material from the primary tumor to allow for protein isolation). Two control non-ELR HNSCC patients were

also selected for comparison, UM-OSCC-01 and UM-LSCC-01. Patient UM-OSCC-01 is a 64-year-old Caucasian male smoker with a history of T4aN2bM0 SCC of the oral cavity. Patient UM-LSCC-01 is a 62-year-old Caucasian female smoker with a history of T2N0M0 SCC of the larynx.

Standard western blotting protocols were used. Briefly, flash frozen tumor proteins were lysed by sonification in RIPA buffer. Three micrograms of each protein was used for western blotting. Primary antibodies against GAPDH (Cat. #8804, Cell Signaling Technology, Danvers, MA) and FGFR1 (Cat. #PA5-27139, Thermo-Fisher Scientific, Wayne, MI; 1:1000 dilution) were incubated overnight at 4 C. The membrane was washed and incubated with an anti-Rabbit HRP linked secondary antibody at room temperature for two hours. The proteins were visualized with chemiluminescence.

The Cancer Genome Atlas

Data was collected from the Oncomine Powertool Database which contains pan-cancer data from TCGA.[6,12,13] This identified 292 previously sequenced HNSCC patients. To begin assessing the basic molecular subtypes of HNSCC and to compare the frequency of *FGF/FGFR* family gene mutations, rearrangements and amplifications with other common alterations in HNSCC, we compared *FGF/FGFR* alterations with *NOTCH* pathway disruptions (*NOTCH1, NOTCH2, NOTCH3, NOTCH4, MAML1* and *MAML2*), *CDKN2A* deletions/mutations, *EGFR* amplifications/mutations and *PIK3CA* gain of function mutations.

Statistical Analysis

Chi-square testing was used to determine significance in calculating co-amplification of *FGF3/4/19, FGFR1–4* aberrations with other commonly mutated pathways, and *FGFR1* amplification prevalence in African-American versus other ethnic groups.

RESULTS

Patient UM-ELR-01

Patient UM-ELR-01 is a 50-year-old Caucasian woman with a history of T2N0M0 (stage II) squamous cell carcinoma of the left oral tongue. She was followed closely for a history of oral tongue dysplasia. She was selected as an ELR patient as she is a never-smoker and non-drinker with a HPV-negative HNSCC. She initially underwent resection with bilateral selective neck dissection and free tissue reconstruction with negative margins. She had no adverse pathologic features and therefore did not undergo adjuvant therapy. She suffered an aggressive recurrence 6 months post operatively which was treated with chemo/radiation therapy.

To identify the molecular features of this tumor that may have driven rapid recurrence and to optimize a method for sequencing cancer related genes from FFPE samples, we first performed targeted sequencing on DNA isolated from a diagnostic FFPE block from the recurrent tumor of UM-ELR-01 (the initial tumor did not provide enough tissue for analysis). We performed amplicon based sequencing of all exons from 400 cancer related

genes (AmpliSeq Comprehensive Cancer Panel) using 40ng of FFPE-derived DNA in the Ion Torrent Personal Genome Machine (PGM). For mutations, we identified only one previously observed *TP53 T125M* (COSMIC ID:44988) mutation was found in UM-ELR-01 (variant frequency 15%). Notably, no mutations and copy number variants were identified in other commonly mutated HNSCC genes and pathways (including *NOTCH, CDKN2A, PI3KCA*, and *EGFR*).

Copy number analysis demonstrated a focal, high-level amplification of *FGFR1* on chromosome 8 (Figure 1). Western blot assay further confirmed high protein expression of *FGFR1* in the recurrent tumor cells when compared to non-ELR HNSCCs (UM-OSCC-01 and UM-LSCC-01) consistent with the sequencing results (Figure 2). Next, FISH was performed on both the primary and recurrent tumor, which confirmed gene amplification at the chromosomal level in both (Figure 3).

Extension to TCGA HNSCC Cohort

Next, we sought to extend our observation from ELR patient with relatively few aberrations to TCGA cohorts of HNSCC in order to define prevalence of *FGF/FGFR* family aberrations in the disease. Notably, TCGA consists of mostly non-ELR patients; most patients have a tobacco and alcohol use history (only 3 ELR patients were identified in this cohort, and all were male; Supplemental Table I,II).[6,14] We postulated that *FGF/FGFR* family aberrations could serve as a molecular sub-group of HNSCC, which may be confounded by additional disruptive events that make the disease more aggressive in high-risk patients. Thus, we first assessed DNA sequencing data from a TCGA cohort of 292 primarily non-ELR patients from TCGA.

Interestingly, we found that *FGF/FGFR* genes were deregulated by mutation, rearrangement or amplification in 37.3% (109/292) of all HNSCC tumors sequenced (Figure 4A). There was a 7.9% (23/292) overall aberration rate in at least one of the four *FGF* receptors, with *FGFR1* being the most frequent at 4.5% (13/292). This is in line with previous studies identifying mutations in *FGFR* in large HNSCC cohorts.[6,15] Additional significant aberrations occurred to the *FGF* ligands, which included *FGF3* amplifications at 22.9% (67/292), *FGF4* amplifications at 21.2% (62/292) and *FGF19* amplifications at 22.6% (66/292). Notably, *FGF3, FGF4*, and *FGF19* are located in an amplicon (along with *CCND1*) on chromosome 11q13.

Because the precise role of *FGF*s in HNSCC pathogenesis is not yet understood, we focused on using the HNSCC TCGA data to stratify *FGFR1–4* and *FGF* genomic alterations with other common genetic aberrations in HNSCC including the *NOTCH* signaling pathway, *CDKN2A, PI3KCA* and *EGFR*. Overall, mutational frequencies were similar between the *FGFR1–4* altered cohort and the non-*FGFR* altered cohort in regards to *CDKN2A* (26.1% vs. 33.4%), *NOTCH* pathway (21.7% vs. 27.5%), and *EGFR* (8.7% vs. 9.7%; Figure 4B). Interestingly, we found a significantly lower frequency of *PIK3CA* gain-of-function mutations in *FGFR1–4* altered patients (4.4% vs. 26.4%; P = 0.02). This data suggests that patients with *FGFR* alterations are unique from those with other activating mutations and that these patients trend towards mutually exclusive from patients with activating *PIK3CA* mutations.

Subsequently, we analyzed *FGF/FGFR* dysregulation in other common cancers from TCGA in order to determine the potential involvement of this pathway across cancer types. Overall, *FGF/FGFR* dysregulation was identified in 28.0% of colorectal adenocarcinomas (73/261), 34.3% of cutaneous melanomas (106/309), and 6.4% of prostate carcinomas (16/249; Figure 5A). Notably, amplifications in *FGFR1* and the *FGF3/4/19* amplicon were more prevalent in HNSCC (Figure 5B; Supplemental Figure 1). Using *FGF19* as a marker of the *FGF3/4/19* amplicon, we examined RNA expression in relation to gene amplification. We did not find a correlation between *FGF19* amplification status and RNA levels (Supplemental Figure 2A). Interestingly, however, *FGF19* RNA expression in HNSCC is elevated relative to prostate and cutaneous melanoma (Supplemental Figure 2B).

We then further assessed the individual gene and combined *FGF/FGFR* pathway correlations with clinical data from the TCGA data set. Interestingly, our analysis revealed that *FGFR1* amplification was more frequent in African Americans. Data showed that 13.8% (4/29) of African American patients were found to have *FGFR1* amplifications while only 3.1% (8/259) of Caucasian patients possessed the aberration ($P < 0.01$; Supplemental Figure 3). Further analysis of TCGA clinical correlates revealed that disruptive events in the *FGF/FGFR* genes were not significantly correlated with age, sex, smoking status, HPV status, TNM stage or grade in this cohort.

DISCUSSION

The fibroblast growth factor receptors (*FGFR*) are a receptor tyrosine kinase family that consists of four highly related genes (*FGFR1–4*).[4] The FGF family further consists of 22 ligands (*FGF*s) that bind with high-affinity to the *FGFR*s.[16] *FGF* binding leads to *FGFR* dimerization, receptor auto-phosphorylation, and activation of downstream signaling pathways[17], including those involved in cell growth, differentiation, metabolism and oncogenesis.[18] Notably, downstream pathways overlap with *EGFR* signaling pathways.

Disruption of normal *FGF* family functions has been linked to other cancers.[4] These include non-small cell lung carcinoma (22% amplification of *FGFR1* and 5% mutation in *FGFR2*)[19–21], pancreatic carcinoma (50–70% amplification of *FGFR1,3* or *4)*[22,23], thyroids carcinoma (*FGFR1* and *FGF2*)[24,25], gastric carcinoma (10% amplification of *FGFR2*)[26,27], bladder carcinoma (40–50% mutation of *FGFR3*)[28], and breast carcinoma (15% amplification of *FGFR1*).[29,30] Furthermore, *FGFR1* amplification has been established as an independent poor prognostic factor for the survival of breast cancer patients with estrogen receptor positive tumors.[31]

At this time, the role of the *FGF/FGFR* pathway is not well understood in HNSCC. Our index ELR patient demonstrated a putative driver amplification of *FGFR1*, suggesting a role for this receptor in HNSCC. Subsequent TCGA analysis of 292 patients demonstrated that *FGF/FGFR* family genes were deregulated by mutation, rearrangement or amplification in over 37% of all HNSCC tumors sequenced. We found an overall rate of 7.9% for *FGFR* aberrations with *FGFR1* amplification being the most common (4.5%).

FGF ligand amplification was most prevalent in *FGF3* (22.9%), *FGF4* (21.2%) and *FGF19* (22.6%). These three *FGF* ligands are on usually co-amplified (with the proto-oncogene *CCND1*) on the chromosome 11q13 locus.[32,33] Because these genes are co-amplified, *FGF3/4/19* amplification and overexpression may play a critical role in the pathogenesis of 11q13-amplified tumors. It is possible that one of the ligands *FGF3/4/19* or *CCND1* alone, or a combination of more than one gene, act as the driver(s) in this amplicon, with the remaining genes acting as passengers. Future systematic studies and follow-up genetic experiments will be needed to address this postulate.

This is the first time that HNSCC aberrations spanning the entire *FGF* family has been reported in the literature. While *FGFR1* amplification has been previously reported, this is the first time that a racial variance has been described, with a higher frequency of *FGFR1* amplifications in the African American population (13.8%) than the Caucasian population (3.1%). These findings warrant further investigation of independent cohorts to confirm this association.

Encouragingly, multiple orally available *FGFR* inhibitors are currently under evaluation in phase II and III trials in breast, thyroid, gastric, bladder and renal cancers.[4,34] The aberrations identified in the *FGF* pathway in a subset of HNSCC suggests that these tumors may be susceptible to targeted pathway inhibition. This is particularly alluring as the majority of these are gene amplifications, suggesting overactivity of the *FGF/FGFR* pathway. Investigation into specific *FGFR* inhibitors via clinical trials and precision medicine programs is warranted, particularly in cases of recurrent or advanced disease in which traditional treatment options have been exhausted.[35] Furthermore, further translational and clinical trial investigation into combinations of receptor tyrosine kinase (*EGFR, HER2, FGFR*) inhibition is alluring due to the fact that these receptors activate common downstream targets.[36]

Together, our data suggests that *FGF/FGFR* signaling is amplified in a subset of HNSCC patients. This new information suggests a rational methodology to leverage ELR-HNSCC patients to define molecular subsets of high risk HNSCC. It further provides a foundation for molecular stratification and advancement of precision medical pre-clinical and clinical trials of FGFR inhibitors in HNSCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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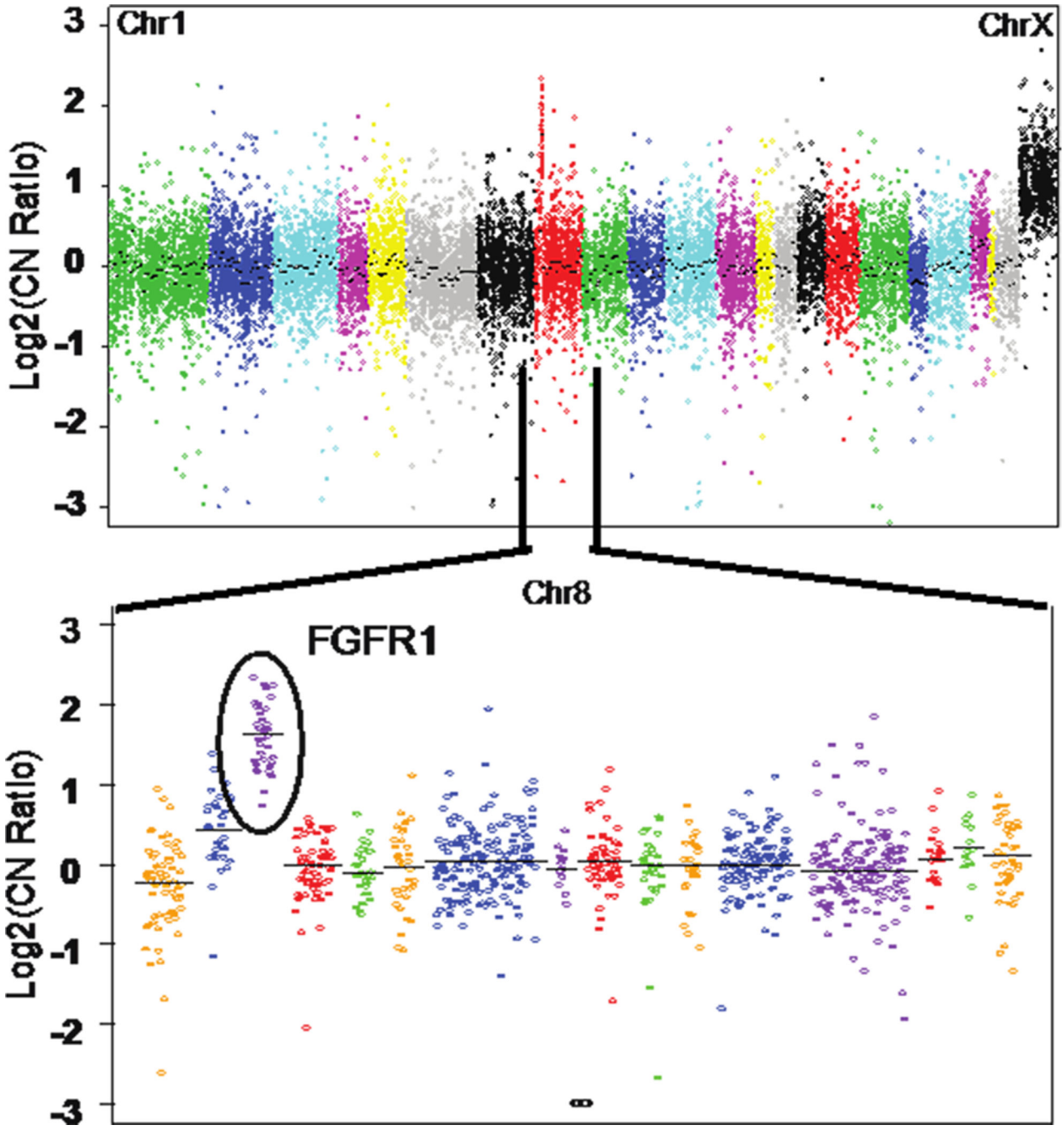


Figure 1. Targeted Next Generation Sequencing identifies *FGFR1* amplification in an ELR tumor

The plot shows relative DNA copy number from an AmpliSEQ analysis of an FFPE tumor sample from UM-ELR-01. The top panel shows genes assessed across the genome. Chromosome 1 is shown on the left and Chromosome X is shown on the right. The X chromosome shows an apparent 1 copy gain as pooled male normal DNA is used as the reference. The bottom panel shows a zoomed in image of Chromosome 8 and an *FGFR1* copy number increase.

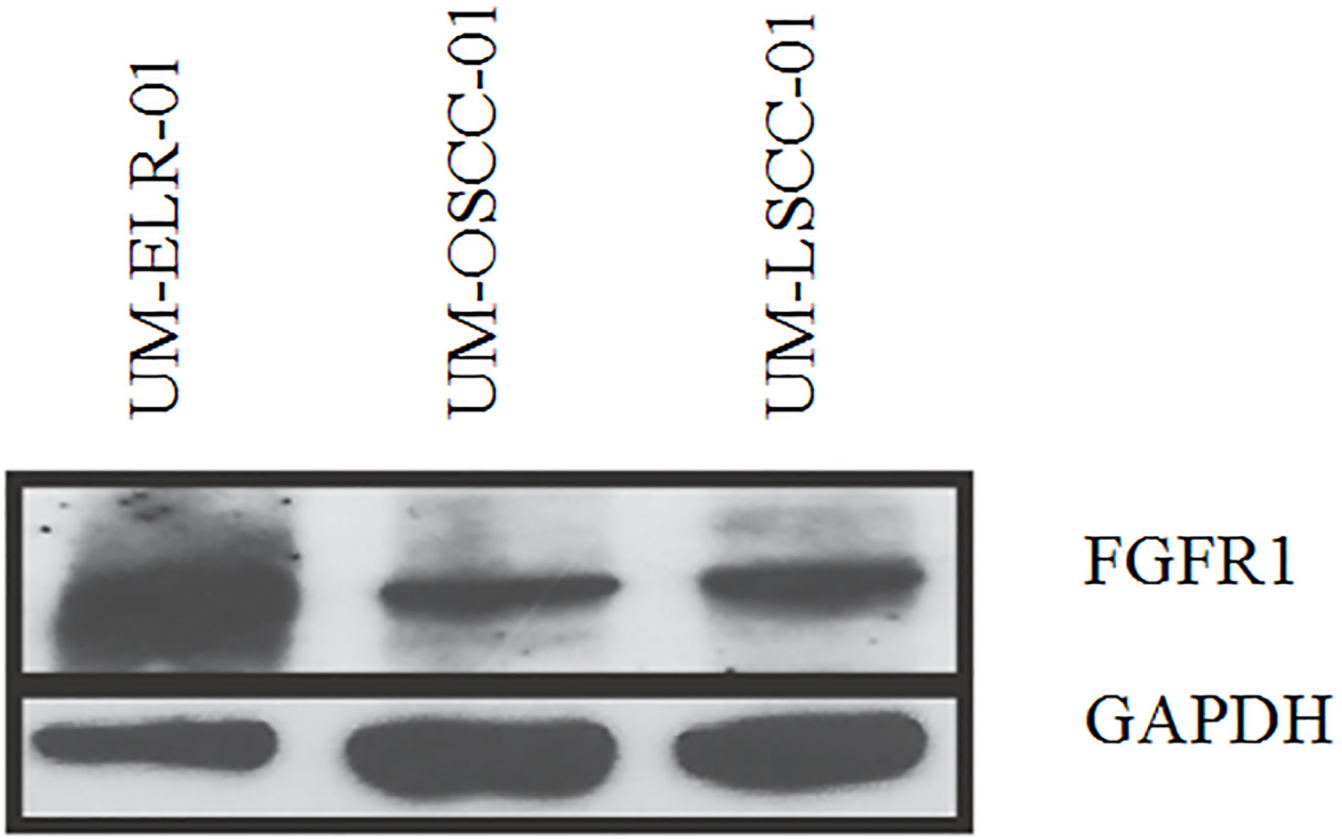
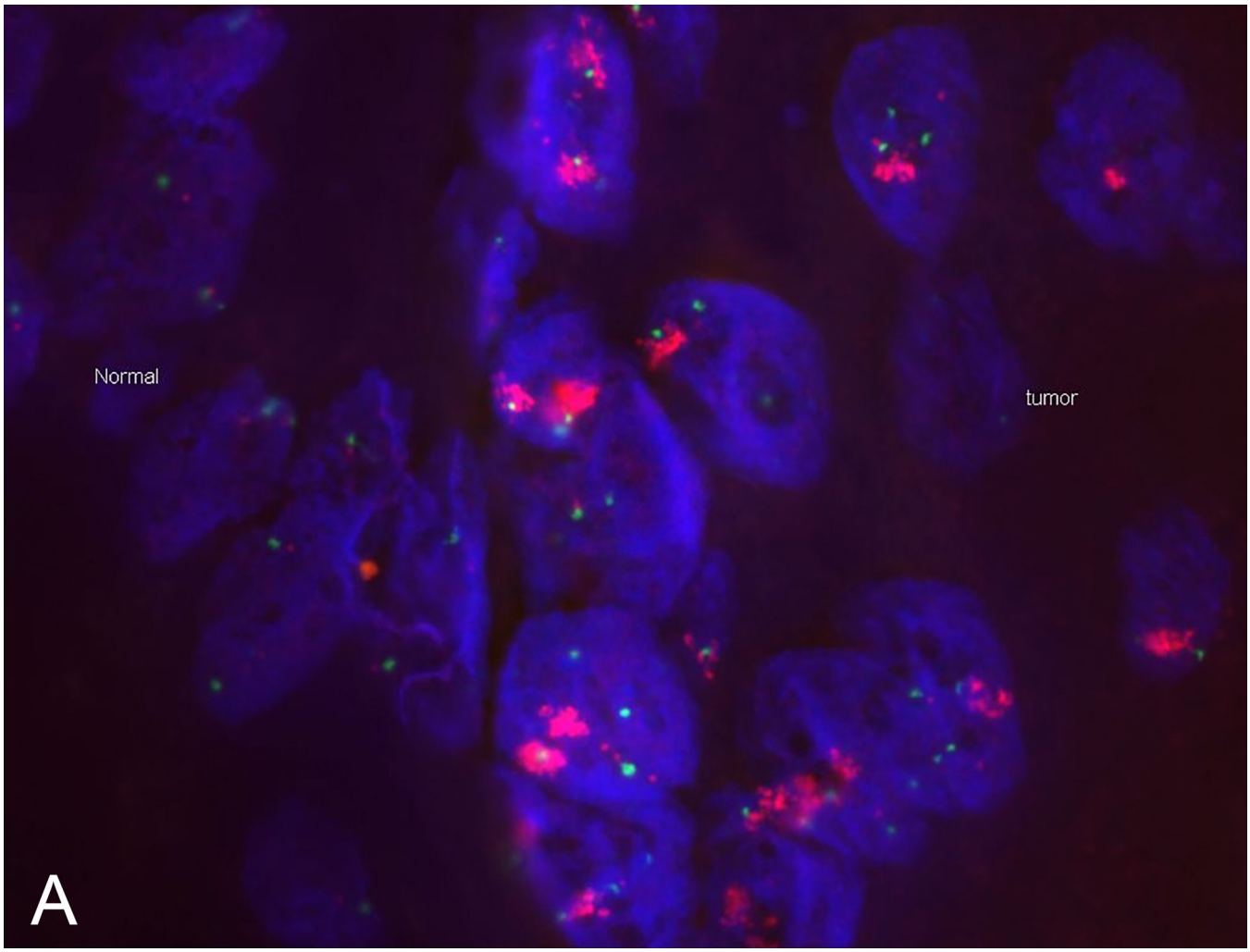


Figure 2. Western blot identifies FGFR1 protein amplification in an ELR tumor

Western blot analysis was performed on 3 HNSCC patients. Our index patient, UM-ELR-01, was compared to two controls, UM-OSCC-01 and UM-LSCC-01. UM-OSCC-01 is a 64-year-old male with a history of T4aN2bM0 SCC of the oral cavity and UM-LSCC-01 is a 62-year-old female with a history of T2N0M0 SCC of the larynx. Even with a lower loading signal, UM-ELR-01 demonstrates higher FGFR1 protein in comparison to the controls.



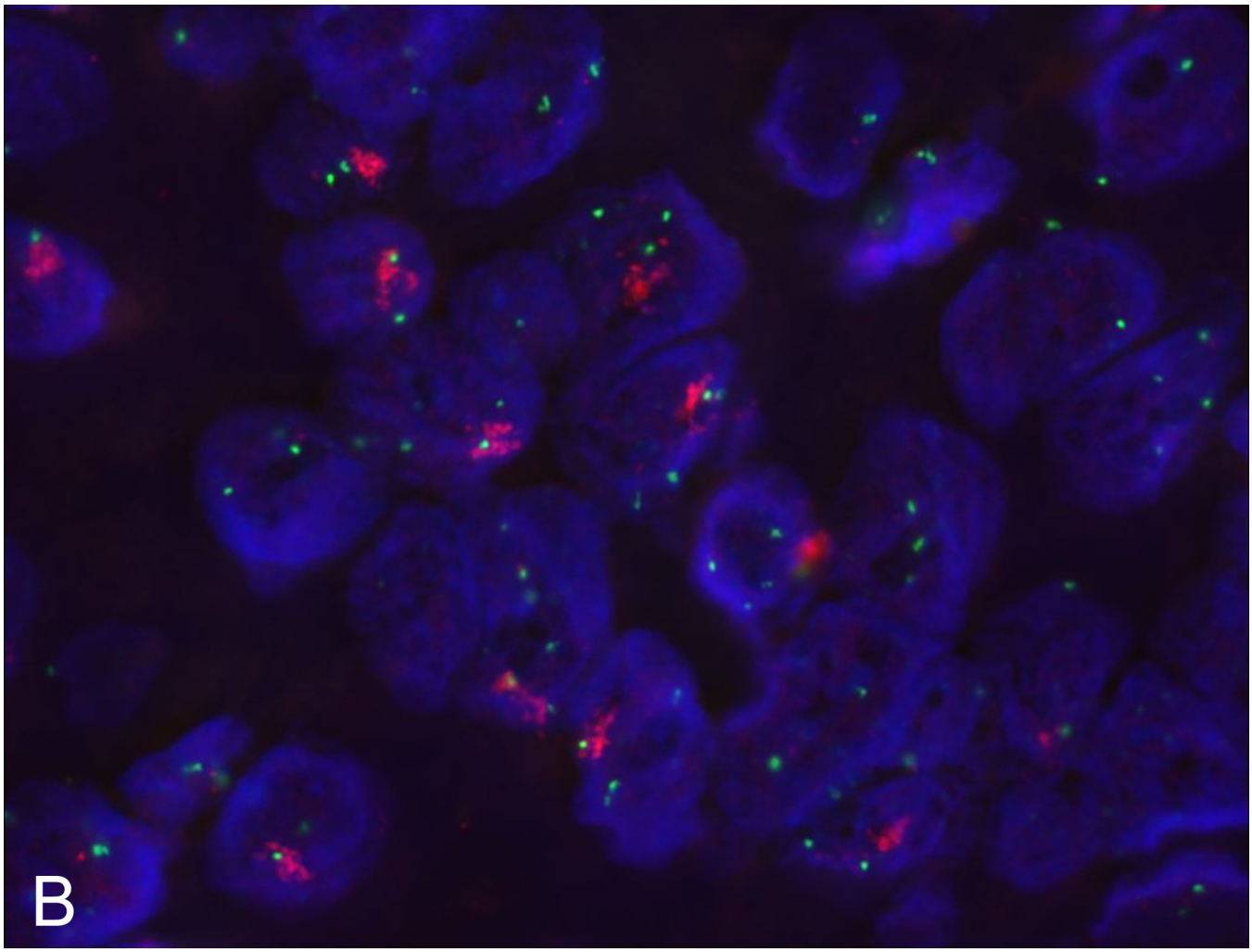


Figure 3. Flourescence in situ hydridization (FISH) identifies *FGFR1* amplification

FISH confirms *FGFR1* amplification at the DNA level in our UM-ELR-01 patient's primary tumor (A) as well as recurrence (B). Red denotes *FGFR1* and green denotes centromere 8.

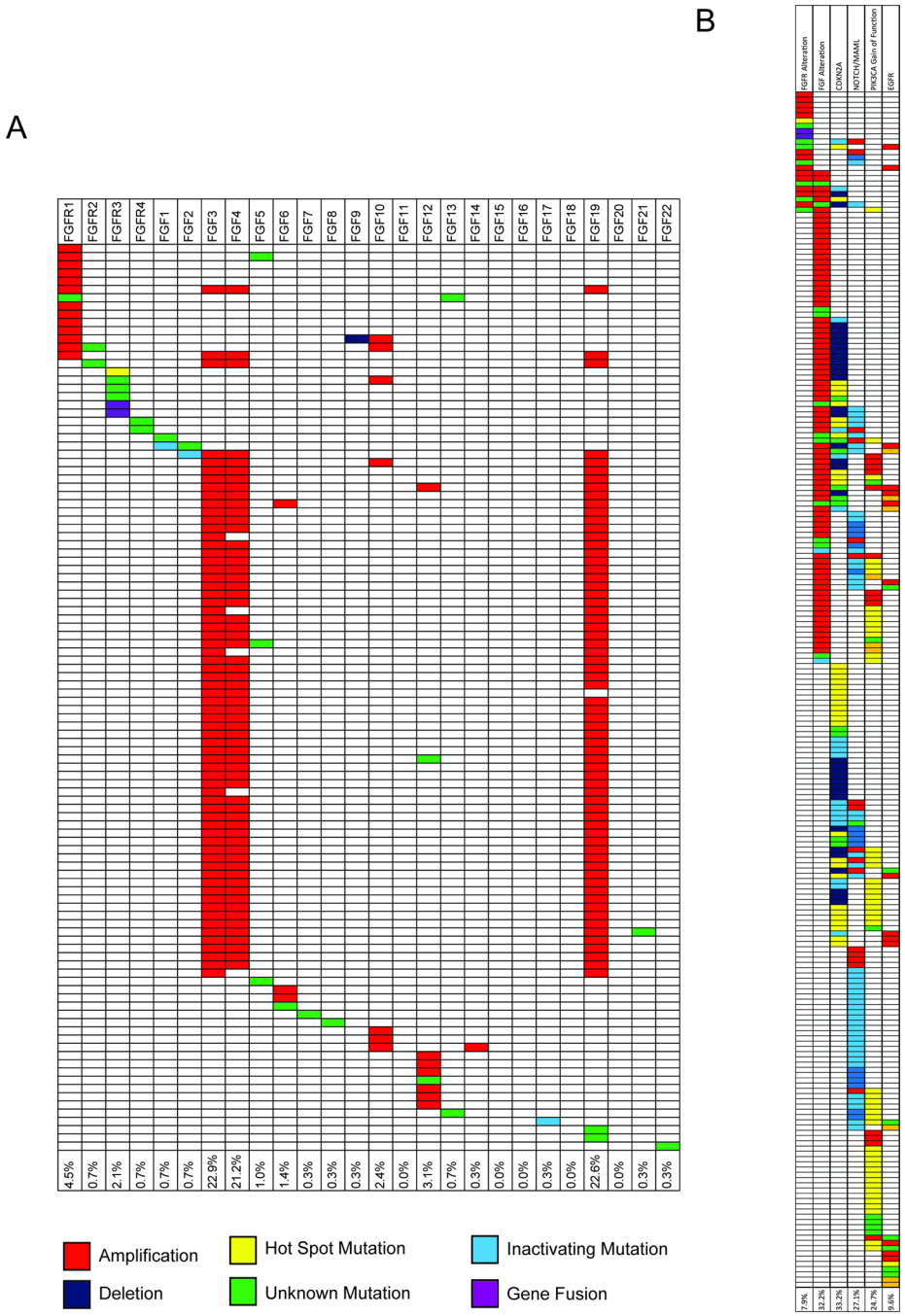


Figure 4. Analysis of genomic aberrations from the TCGA HNSCC data

Individual patients are shown along the Y-axis and *FGF/FGFR* genes are shown along the X-axis. Aberrations are indicated by color code. The table demonstrates a high frequency of *FGFR1, FGF3, FGF4* and *FGF19* aberrations (A). Comparison of trends in patient mutations between *FGF/FGFR* and other commonly mutated pathways (B).

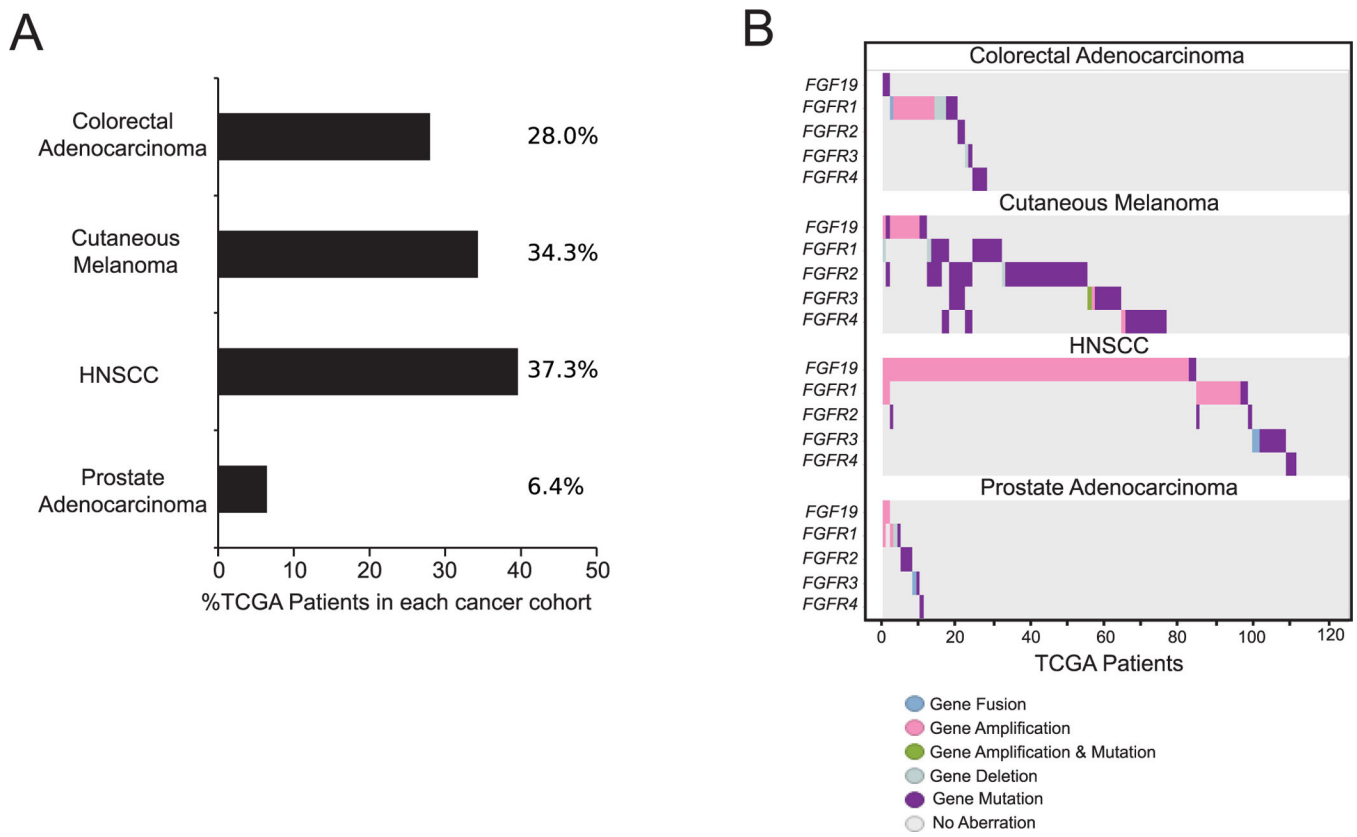


Figure 5. Comparison of *FGF/FGFR* pathway alterations across cancer types

Prevalence of *FGF/FGFR* pathway dysregulation is noted across colorectal adenocarcinoma (28.0%), cutaneous melanoma (34.3%), HNSCC (37.3%), and prostate carcinoma (6.4%; A). Specific genetic dysregulations in each cancer type is shown (B).