Comprehensive Molecular Profiling of Olfactory Neuroblastoma Identifies Potentially Targetable FGFR3 Amplifications

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Abstract

Olfactory neuroblastomas (ONBs), also known as esthesioneuroblastomas, are malignant round-cell tumors that represent up to 5% of sinonasal malignancies. Despite their aggressive course, molecular studies of ONBs have been limited, and targeted therapies are lacking. To identify potential oncogenic drivers and targetable pathways in ONBs, we characterized 20 ONBs, including archived ONBs profiled by targeted, multiplexed PCR (mxPCR)-based DNA next-generation sequencing (NGS) of the coding sequence of over 400 cancer-relevant genes (n = 16), mxPCR-based RNA NGS of 108 target genes (n = 15), and 2 ONBs profiled by comprehensive hybrid-capture–based clinical grade NGS of >1,500 genes. Somatic mutations were infrequent in our cohort, with 7 prioritized nonsynonymous mutations in 5 of 18 (28%) ONBs, and no genes were recurrently mutated. We detected arm/chromosome-level copy-number alterations in all tumors, most frequently gains involving all or part of chromosome 20, chromosome 5, and chromosome 11. Recurrent focal amplifications, often but not exclusively in the context of arm-level gains, included CCND1 [n = 4/18 (22%) tumors] and the targetable receptor tyrosine kinase FGFR3 [n = 5/18 (28%) tumors]. Targeted RNA NGS confirmed high expression of FGFR3 in ONB (at levels equivalent to bladder cancer), with the highest expression observed in FGFR3-amplified ONB cases. Importantly, our findings suggest that FGFR3 may be a therapeutic target in a subset of these aggressive tumors.

Implications: ONBs harbor recurrent chromosomal copy-number changes, including FGFR3 amplification associated with overexpression. Hence, FGFR3 may represent a novel therapeutic target in these tumors. Mol Cancer Res; 1–7. ©2017 AACR.

Introduction

Olfactory neuroblastomas (ONBs), also known as esthesioneuroblastomas, are malignant round-cell neuroectodermal tumors which represent up to 5% of sinonasal malignancies (1). Patients of all ages are affected (range, 3–90 years; ref. 2). Clinical course is variable, with some tumors displaying indolent behavior and others aggressively invading the intracranial cavity and/or displaying metastatic spread to lymph nodes, lung, or bone (1, 2). Five-year survival is less than 50% for tumors with extension beyond the sinonasal cavity (2). Prognostic features are controversial (3), with patient age, tumor grade, presence of lymph node metastases, and TP53 mutations reported as being associated with poor outcome (4). Critically, management of aggressive disease is challenging due to a lack of recurrent, targetable oncogenic drivers, in part because molecular studies of ONBs have been limited (4).

Cytogenetic studies suggest that ONBs are karyotypically complex neoplasms with chromosomal instability; however, recurrent drivers or therapeutic targets have not been consistently nominated (4–10). More comprehensive molecular approaches, such as next-generation sequencing (NGS), have only been reported for three cases of metastatic ONB (including one with matched primary tumor; refs. 11–13). Hence, here we sought to comprehensively profile ONB through NGS to identify recurrent driving somatic alterations and potential therapeutic targets.

Materials and Methods

Case selection

This study was conducted according to previously approved University of Michigan Institutional Review Board Protocols. Retrospective ONB cases were identified by searching University of Michigan Department of Pathology databases for “olfactory neuroblastoma” or “esthesioneuroblastoma.” Diagnosis was confirmed by board-certified Anatomic Pathologists (P.W. Harms...
and J.B. McHugh). Of 48 cases identified, 25 were excluded due to insufficient quality or quantity of material. Therefore, only 23 ONBs were sequenced.

Targeted multiplexed PCR–based NGS

An hematoxylin and eosin–stained section was used as a guide for dissection from a minimum of 4 formalin-fixed, paraffin-embedded (FFPE) 10-μm sections by a board-certified pathologist (F.W. Harms) to obtain a minimal estimated tumor purity of 60%. The areas with highest tumor purity were macrodissected using a scalpel. DNA and RNA were coisolated using the Qiagen Allprep FFPE DNA/RNA Kit (Qiagen) and the Qiagen QIAcube (Qiagen), according to the manufacturer’s instructions and were quantified using the Qubit 2.0 fluorometer (Life Technologies).

Targeted multiplexed PCR (mxPCR)–based NGS was performed as previously described (14–16). Forty nanograms of DNA per sample were used to generate libraries using the Ion AmpliSeq Library Kit 2.0 (Life Technologies) and targeted multiplexed PCR with barcode incorporation using the Comprehensive Cancer Panel (CCP), which targets 1,688,650 bases from 15,992 amplicons representing 409 cancer genes (http://tools.invitrogen.com/downloads/cms_103573.csv). Template preparation was performed using the Ion PI-Hi-Q Template OT2 200 Kit (Life Technologies) and the Ion OneTouch ES Instrument (Life Technologies). NGS of multiplexed templates was then performed on Ion Proton P1 chips using the Ion P-HiQ Sequencing 200 Kit according to the manufacturer’s instructions.

Variant calling and prioritization

Data analysis was performed using in-house–developed, previously validated pipelines using Torrent Suite 4.0.2, with alignment by TMAP and variant calling using the Torrent Variant Caller plugin (17). Annotated variants were filtered to remove synonymous or noncoding variants, poorly supported calls/sequencing artifacts, and germline alterations. Any variant present in the 1000 Genomes (Phase II), Exome Sequencing Project callset, or the ExAC database (http://exac.broadinstitute.org) at population allele frequencies greater than 0.1% was considered germline and excluded. In addition, variants that were present in ExAC with a variant fraction between 40% and 60% (or >90%) were also excluded unless occurring at a well-supported somatic mutation hotspot in COSMIC. All retained variants had flow-corrected variant allele containing reads (FAO) counts ≥ 6 and flow-corrected read depth (FDP) ≥ 20, with overall variant fraction (FAO/FDP) ≥ 10%. In addition, any variant called in >4% of internally sequenced samples using the same panel and not reported in COSMIC, as well as, variants with extreme skewing of forward/reverse flow-corrected reads (FSAF/FSAR <0.2 or >5) were removed. High confidence somatic variants passing the above criteria were then visualized using the Integrated Genomics Viewer for read level confirmation. After filtering and visual confirmation, potential driving alterations were prioritized using the COSMIC Database, with prioritization of recurrently reported variants (>2 occurrences) in oncogenes, and recurrent or deleterious [nonsense, splice site, frame-shifting insertions/deletions (indels)] variants in tumor suppressors.

Copy-number analysis

Normalized, GC-content–corrected read counts per amplicon for each sample were divided by those from a pool of normal male genomic DNA samples (FFPE and frozen tissue, individual, and pooled samples), yielding a copy-number ratio for each amplicon. Gene-level copy-number estimates were determined as described previously by taking the coverage-weighted mean of the per-probe ratios, with expected error determined by the probe-to-probe variance (15, 18). Genes with a log2 copy-number ratio estimate of <−1 or >0.80 were considered to have high-level loss (deletion) and gain (amplification), respectively.

Mi-Oncoseq

Two ONB cases were also identified from the Mi-Oncoseq program at the University of Michigan, which performs comprehensive germline and somatic sequencing for adult patients with advanced cancers or unusual presentations to facilitate clinical trial enrollment and guide precision medicine approaches. Clinical grade, hybrid-capture–based exome or targeted (1,711 cancer-related genes) sequencing of tumor and normal tissue to identify somatic mutations, fusions, and copy-number alterations (CNA) was performed as described (19). Potential driving somatic mutations were classified as for targeted sequencing described above.

Expression profiling by RNAseq

Targeted mxPCR-based NGS of RNA was performed on coisolated RNA from all 23 ONB samples (ONB1-23) using a custom Ion AmpliSeq panel assessing 103 target genes and 8 housekeeping genes relevant for urothelial carcinoma, as previously described (16), except template preparation and sequencing, which were performed as described above for DNA mxPCR. Data analysis was performed essentially as described using the Coverage Analysis RNA Plug-in (16). Samples with low-quality data (<150,000 total mapped reads or <50% full-length reads) were excluded from all further analyses. For high-quality samples, for each amplicon, full-length read counts were log2 transformed (read count + 1). Then, to determine normalized expression for each target gene, the log2 count was normalized to the median of the log2 counts (geometric mean) of five housekeeping genes expressed across normal urothelium, urothelial cancer, and ONB (CTCF, TARDBP, HNRNPK, TRHAP3, SAFB). Data from previously sequenced normal urothelium (n = 3) and urothelial carcinoma (n = 11; ref. 16) meeting the above quality criteria, as well as urothelial carcinoma with extensive squamous differentiation (n = 2; Hovelson and colleagues, manuscript in preparation) were processed exactly as for ONB samples. Unsupervised centroid linkage hierarchical clustering of genes and samples (with median centering of genes) was performed using Cluster 3.0 and visualized using Java TreeView. Median normalized expression of FGFR3, FOXA1 (luminal subtype marker), and KRT6A (basal subtype marker) was compared across luminal subtype bladder cancers, basal subtype bladder cancers, and ONB by the Kruskal–Wallis test with pairwise comparisons if results were statistically significant (P < 0.05) using MedCalc v14.12.

Immunohistochemistry

Immunohistochemical staining was performed on a Ventana automated stainer, with staining conditions as described in Supplementary Table S1.

Results

ONB cohort

We identified a cohort of 23 ONBs with sufficient available archived FFPE tissue for targeted mxPCR NGS. Seven and eight samples showed poor-quality DNA and RNA sequencing.
(RNAseq) data, respectively, and were excluded from subsequent analysis, resulting in a final cohort consisting of 18 tumors sequenced in total from unique patients (Supplementary Table S2). The mean patient age was 53 years (median, 55; range, 26–72 years), with samples from 14 men and 4 women. The tumors were mainly primary from the sinonasal region (n = 9); however, there were also recurrent (n = 5) as well as locoregional metastases (4). There was a prior history of radiotherapy for seven tumors (three recurrences and four metastases). We also identified two ONBs profiled as part of the Mi-Oncos eq clinical sequencing program at the University of Michigan to identify precision medicine opportunities for patients with advanced cancer. Both samples were locoregional recurrences from male patients ages 40 to 59 who had been treated with radiotherapy [one with radiotherapy and chemotherapy (cisplatinum/etoposide)]. Considering both archival and Mi-Oncoseq cases, the final cohort size was 20 tumors.

NGS demonstrates minimal recurrent driving somatic mutations in ONBs

Targeted mxPCR NGS of DNA assessing the coding sequence of 409 cancer-related genes from the 16 informative archived samples generated an average of 4,529,551 mapped reads yielding 263x targeted base coverage (Supplementary Table S3). After stringent filtering (see Materials and Methods), we identified an average of 7 high-confidence somatic mutations (range, 0–21) and <1 high-confidence prioritized driving somatic mutation (range, 0–2) across the 16 samples (Supplementary Table S4). In the two Mi-Oncos eq–profiled ONBs, we identified 16 (from >1,700 targeted genes) and 45 somatic mutations (from full-exome capture), respectively, with no prioritized driving somatic mutations. All prioritized somatic mutations from the 18 total informative samples are shown in Fig. 1 and listed in Supplementary Table S4.

No genes showed recurrent prioritized somatic mutations, and six of the seven prioritized somatic mutations were in tumor suppressors. For example, ONB16 (a case from a patient who died of disease) harbored a TP53 V272M mutation and a deleterious PTEN Y16X mutation (Fig. 1; Supplementary Table S4). We confirmed the expected p53 protein overexpression in this case by immunohistochemistry (Supplementary Fig. S1). Likewise, we also identified inactivating mutations of the lysine methyltransferase genes KMT2C Y366X (MLL3) in ONB 15 and KMT2A S3702X (MLL1) in ONB6 (Fig. 1; Supplementary Table S4).

Figure 1.
Integrated heatmap of prioritized mutations and copy-number changes in olfactory neuroblastomas (ONBs) identified by comprehensive NGS. Integrated table of high-confidence, prioritized somatic mutations, and CNAs identified by NGS of >400 cancer-related genes across a cohort of 18 ONBs. Clinicopathologic information for each sample is shown above the heatmap according to the legend (XRT = radiation therapy; Chemo = chemotherapy). All prioritized somatic mutations and high-level CNAs are shown in the heatmap with alteration type indicated by cell color according to the legend. FP indel, frame-preserving insertion/deletion; FS indel, frame-shift insertion/deletion; Loss, homozygous copy-number loss; Gain, copy-number gain.
ONB-06 harbored an activating CTNNB1 S37F mutation, and we confirmed the expected nuclear beta catenin expression by immunohistochemistry (Fig. 1; Supplementary Fig. S2; Supplementary Table S4). Taken together, these results suggest that driving somatic mutations in known cancer associated genes are infrequent in ONB.

ONBs harbor highly recurrent chromosome/arm-level CNAs and amplifications in FGFR3 and CCND1

Although driving somatic mutations were rare in our cohort, we found that all ONBs harbored multiple whole chromosome (or arm level) CNAs, including highly recurrent gains and losses (Fig. 2A and B). Chromosome 20 gains were the most frequent CNA gains observed in our cohort, present in 16 of 18 (89%) ONBs. Gains of chromosomes 5, 7, and 11 were similarly common in our cohort (occurring in 78%, 61%, and 67% of tumors, respectively). Losses of chromosomes 12, 8, 3, and 1 were also frequently observed. In addition to these single-copy chromosome-level gains and losses, we also identified recurrent low-level amplifications of known oncogenes in our ONB cohort (Figs. 1 and 2), including CCND1 in 4 of 18 (22%) samples. Most intriguingly, we identified focal low-level amplifications of the targetable receptor tyrosine kinase FGFR3 in 5 of 18 (28%) ONBs (Fig. 2 and Supplementary Table S5). Two samples harbored coamplification of CCND1 and FGFR3 (Figs. 1 and 2C), and FGFR3 amplification was also observed in the absence of more broad chromosome 4 gain (Fig. 2C). Focal, high-level deletions were rare in our cohort, with the only prioritized two-copy deletion in our cohort occurring in ONB-2194, which harbored a focal, two copy deletion of CDKN2A (and CDKN2B), as shown in Fig. 2B.

Expression profiling of ONBs confirms high FGFR3 expression in amplified cases

To assess the expression of relevant genes in ONB, most notably FGFR3, we profiled coisolated RNA from the 23 ONBs subjected to DNA mxPCR sequencing by targeted mxPCR RNAseq using a previously described custom Ampliseq panel assessing 103 target genes (and 8 housekeeping genes) relevant for urothelial carcinoma (16). Importantly, this panel assesses FGFR3, enabling us to compare the expression profiles our current ONB cohort to our previously profiled luminal (which is defined in part by high FGFR3, FOXA1, and KRT19 expression; ref. 20) and basal subtypes of urothelial carcinoma (defined in part by high KRT6A and KRT14 expression; ref. 20). Fifteen of the 23 ONB samples generated high-quality mxPCR RNAseq results, with ONB-04,
ONB-15, and ONB-10 only having high-quality DNA sequencing data, and ONB-14 and ONB-01 only having high-quality RNAseq data. Across the 15 ONB samples, we generated an average of 2,593,832 total mapped reads and 2,132,101 full-length reads (77%; Supplementary Table S6). Unsupervised hierarchical clustering of the high-quality ONB samples and previously profiled normal urothelial samples showed clear separation of ONB from all urothelial samples (n = 3, normal urothelial samples; n = 7, luminal bladder cancers; n = 2, luminal/basal bladder cancers; and n = 4, basal bladder cancers), as shown in Fig. 3A. Likewise, ONB showed essentially no expression of nearly all canonical luminal and basal subtype markers (Fig. 3A and B). For example, normalized log2 expression of FOXA1 (luminal marker) differed substantially across luminal bladder cancer, basal bladder cancer, and ONB (Kruskal–Wallis test P = 0.0001, median log2 expression 3.4, −0.2, and −9.2, respectively; all pairwise comparisons P < 0.05; Fig. 3B), as did expression of the basal marker KRT6A (Kruskal–Wallis test P = 0.0002, median log2 expression 2.4, 10.8, and −5.6, respectively; all pairwise comparisons P < 0.05; Fig. 3B). Strikingly, however, ONB showed nonsignificantly greater FGFR3 expression than luminal or basal bladder cancers (median log2 expression 0.1 vs. −1.2 and −1.4, respectively; Kruskal–Wallis test P = 0.21; Fig. 3B), and the highest FGFR3 expression in the cohort was observed in ONB-12 (Fig. 3A), which harbored a high-level FGFR3 amplification by DNA mxPCR–based sequencing. Likewise, ONB-18, which also had an FGFR3 amplification by DNA sequencing, showed...
the fifth highest FGFR3 expression across the 31 total samples and had the second highest FGFR3 expression in the ONB cohort (Fig. 3A). Lastly, although FGFR3 is activated in some tumor types by both hotspot mutations and gene fusions (21), we did not observe any activating mutations by DNA sequencing, and the Mi-Oncoseq cases, which underwent capture transcriptome sequencing, did not harbor any driver gene fusions (including those involving FGFR3). Taken together, these results demonstrate relatively high expression of FGFR3 across ONB and confirm that the highest expression is observed in cases with focal FGFR3 amplification.

Discussion

ONBs are aggressive tumors for which accurate prognostication is challenging and targeted therapeutic strategies are lacking. Here, we utilized multiple NGS approaches on a cohort of 23 total ONBs to determine the genomic landscape, identified potential therapeutic strategies, and performed limited transcriptional profiling to confirm DNA findings. We are aware of three case reports sequencing ONBs (three metastatic ONBs, one with a matched primary). In the study of a matched primary and metastatic tumor, across ONB and confirming the definition of these results demonstrate relatively high expression of FGFR3

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Disclosure of Potential Conflicts of Interest

S. A. Tomlins is a cofounder, consultant for, and laboratory director of Strata Oncology; reports receiving commercial research grant from Compendia Bioscience/Life Technologies/Thermo Fisher; and has provided expert testimony for Thermo Fisher (Travel Support). No potential conflicts of interest were disclosed by the other authors.
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