Differential Gene Expression and Pathway Analysis in Juvenile Nasopharyngeal Angiofibroma Using RNA Sequencing

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Abstract
Juvenile nasopharyngeal angiofibroma (JNA) is a highly vascularized and locally aggressive tumor that typically presents in adolescent males. The molecular biology of this tumor remains understudied. We sought to identify differentially expressed genes in the JNA transcriptome through messenger RNA sequencing of primary fibroblasts from 2 tumor explants and tonsil tissue from tumor-free subjects. In total, 1088 significant, differentially expressed genes were identified with 749 upregulated and 339 downregulated. Pathway analysis identified a number of activated signaling pathways, most notably, the vascular endothelial growth factor (VEGF) pathway (adjusted overlap \( P = .03 \)). VEGF-A showed a 4.4-fold upregulation in JNA samples. In addition, the angiogenic receptor, fibroblast growth factor receptor 2 (FGFR2), was not present in tumor-free samples but increased in JNA. We validate these findings with immunohistochemistry, demonstrating upregulation of VEGF and FGFR2 in patient sections. Inhibition of the VEGF or FGFR signaling axes may have therapeutic potential in the treatment of JNA.

Keywords
juvenile nasopharyngeal angiofibroma, RNA sequencing, transcriptome, vascular endothelial growth factor

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uvenile nasopharyngeal angiofibroma (JNA) is a benign yet aggressive tumor that typically presents in adolescent males with nasal obstruction and epistaxis.1,2 Surgical resection provides the primary therapy. However, recurrence rates reach up to 20%.3,4 Histologically, JNA is a vascular, pseudocapsulate lesion composed of stroma that is predominantly fibroblasts and dense collagen matrix.5 The current biological understanding of JNA is based on a limited number of factors assessed by polymerase chain reaction or immunohistochemistry (IHC).6 As such, we sought to build upon the current understanding of the underlying pathophysiology by assessing transcriptional profiles by RNA sequencing (RNA-seq).

RNA-seq characterizes transcriptome-wide expression in solid tumors.7 The transcriptome describes the subset of genes transcribed and represents functional cellular activity. We established primary cultures from JNA and tumor-free tissue fibroblasts and characterized the JNA transcriptome using RNA-seq to identify signaling pathways in this tumor. We validated these findings in multiple patient samples using IHC.

Methods

Cell Lines
Deidentified JNA and tonsil explants from tumor-free patients were collected by the Biospecimen Repository Core Facility with written informed consent using protocols approved by the institutional review boards at the University of Kansas Medical Center. Primary fibroblasts from 2 JNA tumor explants and 2 tumor-free adult tonsils were derived using our described protocol.8 Other cell types in the tissue did not grow in culture. Fibroblasts were grown

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in Dulbecco’s modified Eagle’s medium (DMEM; Corning, Corning, New York) supplemented with 10% fetal bovine serum (FBS; Sigma, St Louis, Missouri).

**RNA Extraction and Sequencing**

RNA was collected using Trizol (ThermoFisher, Waltham, Massachusetts). Stranded messenger RNA (mRNA) sequencing was performed using the Illumina HiSeq2500 (Illumina, San Diego, California). STAR software mapped data to the human genome (assembly GRCh38.rel77). The sequenced reads were submitted to the Gene Expression Omnibus repository (accession number GSE99247). Cufflinks software generated transcript abundance estimates (fragments per kilobase per million [FPKM] reads). Cuffdiff software calculated differential gene expression.

Ingenuity pathway analysis (IPA; Ingenuity Systems, Redwood City, California) was used to assess pathways of differentially expressed genes with a false discovery rate of ≤0.05 and fold change of ±1.5.

**Immunohistochemistry**

IHC was performed on tissue obtained from patient paraffin samples. Twenty-eight JNA, 8 normal adjacent, and 2 additional samples from tumor-free patients were included (age JNA: median = 16.5 years, interquartile range = 5.75 years; normal: median = 15.5 years, interquartile range = 6 years). Primary antibodies fibroblast growth factor receptor 2 (FGFR2; E10570) and vascular endothelial growth factor (VEGF; E2610) (Spring Bioscience, Pleasanton, California) were used. Immunohistochemical staining was performed using the IntelliPATH FLX Automated Stainer (Biocare Medical, Pacheco, California). Staining intensity (0 indicates no staining, 1 weak staining, 2 moderate, and 3 strong staining) was determined by a board-certified pathologist (O.T.). Significant differences were determined by nonparametric Mann-Whitney U tests (GraphPad Prism 6, version 6.03; GraphPad Software, La Jolla, California).

**Results**

A limited number of factors comprise the current biological understanding of JNA. We sought to build upon current understanding by assessing the JNA transcriptome. RNA sequencing identified 1088 differentially expressed genes with 749 upregulated and 339 downregulated. IPA revealed a number of growth factors implicated in disease progression (Table 1). VEGF-A, a factor previously associated with JNA due to its strong activation of angiogenesis, was the most highly activated growth factor pathway. Other angiogenic

| **Table 1.** Top Upregulated Growth Factors Listed by Ingenuity Pathway Analysis Activation z Scores. |
|--------------------------------------------------|---------------------------------|-----------------|-----------------|-----------------|
| Growth Factor                                    | Gene Symbol                      | Expression Fold Change | False Discovery Rate | Activation z Score |
| Vascular endothelial growth factor A             | VEGF-A                           | 4.4               | .002             | 3.1             |
| Hepatocyte growth factor                         | HGF                              | 3.0               | .03              | 2.9             |
| Transforming growth factor α                     | TGF-α                            | ∞                 | .002             | 2.9             |
| Bone morphogenetic protein 2                    | BMP2                             | 7.9               | .002             | 2.8             |
| Amphiregulin                                     | AREG                             | 25.7              | .002             | 2.6             |
| Epiregulin                                       | EREG                             | 3.5               | .002             | 2.4             |
| Placental growth factor                          | PGF                              | 78.0              | .002             | 2.4             |
| Insulin-like growth factor 1                     | IGFI                             | ∞                 | .002             | 2.3             |

| **Table 2.** Differentially Expressed Genes Associated with Vascular Endothelial Growth Factor Signaling. |
|--------------------------------------------------|---------------------------------|-----------------|-----------------|-----------------|
| Gene Name                                        | Gene Symbol                      | Expression Fold Change | False Discovery Rate |
| Actin, gamma 2, smooth muscle, enteric           | ACTG2                           | −11.4             | .002             |
| Fibroblast growth factor receptor 2             | FGFR2                           | ∞                 | .002             |
| Fibroblast growth factor receptor 4             | FGFR4                           | 8.1               | .002             |
| Fms-related tyrosine kinase 1                   | FLT1                            | −5.2              | .02              |
| Foxhead box O1                                  | FOXO1                           | 30.3              | .002             |
| Placental growth factor                         | PGF                             | 78.0              | .002             |
| Phosphoinositide-3-kinase regulatory subunit 2  | PIK3R2                          | 2.6               | .01              |
| Phospholipase C gamma 2                         | PLCG2                           | 16.4              | .02              |
| Vinculin                                        | VCL                             | −2.3              | .05              |
| Vascular endothelial growth factor A            | VEGF-A                          | 4.4               | .002             |
| Vascular endothelial growth factor C            | VEGF-C                          | −2.1              | .02              |
factors, including transforming growth factor \( \alpha \) (TGF-\( \alpha \)), hepatocyte growth factor (HGF), and placental growth factor (PGF), were also strongly upregulated. Downstream mediators of VEGF indicate pronounced activation of this pathway (Table 2). These findings support previous studies implicating VEGF activity in disease progression.12

Also of interest was the strong upregulation of fibroblast growth factor receptors (FGFRs). JNA expressed FGFR2, which was absent in the normal samples. IPA predicts activation of the FGFR signaling network (z score = 2.2). This builds upon previous understanding that basic fibroblast growth factor (bFGF), a pleiotropic growth factor acting as a mitogen for both fibroblast and endothelial cells, is strongly associated with disease progression.13,14 Thus, our differential expression analysis corroborates current understanding of drivers underlying this disease while also detailing novel pathways not previously associated.

**IHC Validates VEGF and FGFR Induction**

IHC provides a method to validate RNA-seq using additional patient samples. IHC demonstrates upregulation of VEGF in JNA specimens (Figure 1) in corroboration with our RNA-seq findings and past reports. In addition, FGFR2 protein levels were overexpressed in JNA specimens, supporting our RNA-seq identification of FGFR signaling (Figure 1). This is the first evidence of FGFR overexpression in JNA patient specimens and supports targeted therapy against FGFR in JNA patients.14

**Discussion**

Currently, no options exist for JNA-targeted therapy. We characterized the JNA expression profile using RNA-seq to provide a detailed transcriptome characterization and identify novel targets to direct future trials.

Upregulated growth factors are of interest due to the abundance of therapeutics targeted toward growth factor receptors. Previous studies demonstrate VEGF immunostaining in JNA tissue specimens and associate VEGF with increased vascularization and persistent disease.5,15,16 Our results support upregulation of VEGF-A and suggest activation of VEGF signaling with upregulation of downstream mediators. In addition, we provide evidence for targeting the FGFR pathway, also implicated in angiogenesis.

This study’s shortcomings pertain to the rarity of JNA and the small number of specimens we could obtain to culture. As tumors are heterogeneous entities, our analysis may be limited by using a single cell type for sequencing. In addition, differential expression is dependent upon the control group (tonsillar fibroblasts from tumor-free patients).

**Conclusions**

We report differentially expressed genes in JNA using RNA-seq. These data provide novel targets for future study and potentially therapeutic trials. We identify VEGF and FGFR upregulation and predict pathway activation. Inhibition of the VEGF and FGFR signaling axes may have therapeutic potential.

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**Author Contributions**

Joel W. Jones, design and analysis of the work, drafting and revision of the manuscript, final approval of manuscript; Shireen Usman, acquisition of data, analysis of work, revision of manuscript, final approval of manuscript; Jacob New, acquisition and interpretation of data, revision of manuscript, final approval of manuscript; Andrew Holcomb, acquisition and interpretation of data, revision of manuscript, final approval of manuscript; Sumedha Gunewardena, acquisition of data, analysis of work, revision of manuscript, final approval of manuscript; Ossama Tawfik, analyses of immunohistochemistry-stained slides, revision of manuscript, final approval of manuscript; Larry Hoover, conception and design, analysis of data, revision of manuscript, final approval of manuscript; Daniel Bruegger, conception and design, analysis of data, revision of manuscript, final approval of manuscript; Sufi Mary Thomas, conception and design, supervision of data acquisition and interpretation, funding, analysis of data, drafting, revision and final approval of manuscript.

**Figure 1.** Immunohistochemistry demonstrates fibroblast growth factor receptor 2 (FGFR2) and vascular endothelial growth factor (VEGF) expression. Mean staining intensity assessed in juvenile nasopharyngeal angiofibroma (JNA; n = 28) or normal (n = 8 adjacent mucosa and n = 2 tumor-free patients). Error bars ± SEM (×4.0, ×60 for high-power image).
Disclosures

Competing interests: None.

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