Targeted RNA Sequencing of Formalin-Fixed, Paraffin-Embedded Temporal Arteries From Giant Cell Arteritis Cases Reveals Viral Signatures

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Abstract

Background and Objectives
Varicella zoster virus (VZV) antigen has been detected in temporal arteries (TAs) of individuals with giant cell arteritis (GCA), the most common systemic vasculitis in older adults. Thus, we explored the contribution of VZV to GCA pathogenesis.

Methods
Formalin-fixed, paraffin-embedded TA sections from biopsy-positive GCA participants with VZV antigen (GCA/VZV-positive; n = 20) and without (GCA/VZV-negative, n = 20) and from normal participants with VZV antigen (control/VZV-positive, n = 11) and without (control/VZV-negative, n = 20) were analyzed by targeted RNA sequencing of the whole human transcriptome (BioSpyder TempO-Seq). Ingenuity pathway analysis and R-computational program were used to identify differentially expressed genes and pathways between groups.

Results
Compared with control/VZV-negative TAs, GCA/VZV-negative and GCA/VZV-positive TAs were significantly enriched for human transcripts specific for pathways involved in viral infections, including viral entry, nuclear factor kappa B activation by viruses, and other pathogen-related immune activation pathways. Similarly, human gene sets supporting viral infection were found in control/VZV-positive TAs that showed no morphological signs of inflammation, suggesting that the enriched pathways were not nonspecific signatures of infiltrating immune cells. All GCA TAs and control/VZV-positive TAs showed enrichment of transcripts involved in vascular remodeling, including smooth muscle cell migration.

Discussion
The detection of viral and immune activation pathways in GCA TAs supports a role for virus infection in GCA pathogenesis. In addition, the detection of viral pathways in control/VZV-positive TAs, along with vascular remodeling pathways, suggests that these samples may represent early infection with progression to clinical disease, depending on host and other environmental factors.
Glossary

COMIRB = Colorado Multiple Institutional Review Board; FFPE = formalin-fixed, paraffin-embedded; GCA = giant cell arteritis; gE = glycoprotein E; GSEA = Gene Set Enrichment Analysis; HSV = herpes simplex virus; IFN = interferon; IHC = immunohistochemistry; IL = interleukin; TA = temporal artery; TLR = toll-like receptor; TNF = tumor necrosis factor; VZV = varicella zoster virus.

Giant cell arteritis (GCA), the most common systemic vasculitis in the elderly, has been proposed to be a form of extracranial varicella zoster virus (VZV) vasculopathy because both VZV vasculopathy and GCA (1) affect the elderly; (2) can present with vision loss, headaches, and stroke; and (3) produce transmural arterial inflammation with giant/epithelioid cells and medial damage in temporal, cerebral, and/or other systemic arteries. Importantly, 70% of biopsy-confirmed GCA temporal arteries (TAs) contain VZV antigen compared with 18% of controls. Follow-up studies by other groups yielded variable frequencies of VZV antigen, raising the question of whether virus contributes to GCA pathogenesis. Previously, gene expression analyses of GCA TAs that would identify viral signatures were challenging due to the analysis of fresh or frozen tissue. This technology advancement has allowed us to determine the transcriptional profiles of specific VZV antigen–containing regions within single, FFPE GCA TA slides and compare with control TAs to elucidate the role of virus infection in GCA pathogenesis.

Methods

Standard Protocol Approvals, Registrations, and Patient Consents

Archived patient samples were deidentified and deemed exempt from human research as defined by the Colorado Multiple Institutional Review Board (COMIRB) policies and current regulations and in accordance with Office for Human Research Protection and Food and Drug Administration guidelines (Protocol no. 13-2550; COMIRB, 303-724-1055).

Patient Samples

Forty FFPE TA biopsies from patients with GCA older than 50 years were used; all GCA TAs were biopsy-positive with transmural inflammation, giant/epithelioid cells, and medial damage. Controls were 31 normal TAs (without zoster, diabetes, cancer, substance abuse, or immunosuppression) removed postmortem from participants >50 years of age at the University of Colorado Hospital, Arapahoe County Coroner’s Office, and Denver Office of the Medical Examiner. These TAs were previously obtained and analyzed by immunohistochemistry (IHC) and quantitative PCR for the presence of VZV antigen and DNA, respectively. Previously published data showed that of the 40 GCA TAs, 16 contained VZV antigen with corresponding DNA from the scraped VZV antigen-positive fields and 4 contained VZV antigen only (20 GCA/VZV-positive TAs); 20 did not contain VZV antigen (20 GCA/VZV-negative TAs). Of 31 control TAs, 3 contained VZV antigen with corresponding DNA and 8 contained VZV antigen only (11 control/VZV-positive TAs); 20 did not contain VZV antigen (20 control/VZV-negative TAs). Patient sample information is in eTable 1 (links.lww.com/NXI/A574).

FFPE Tissue Sequencing and Analysis

For whole human transcriptome analysis, FFPE slides from TAs were assayed using TempO-Seq–targeted RNA sequencing plates, reagents, protocols, and software (BioSpyder Technologies). TempO-Seq exclusively detects human transcripts; viral transcripts will not be detected. For slides with VZV antigen, the same antigen-containing regions on unstained adjacent slides were measured, scraped (10 mm² pooled regions of interest per participant), and placed into PCR tubes containing 1X lysis buffer. For slides without VZV antigen, equivalent areas of arteries from unstained slides were analyzed. Using a thermocycler, samples were lysed, coded adjacent primer pairs for each specific human transcript were annealed to sample RNA, and primer pairs for each transcript ligated and then amplified per manufacturer’s instructions. If a transcript is present, the adjacent 25-nucleotide primer pairs anneal to their specific target, ligate together, and produce a 50-nucleotide transcript-specific, coded amplicon. Amplified PCR products were pooled into a single library, concentrated using a PCR cleanup kit (Macherey-Nagel, Düren, Germany), and run on the Illumina NextSeq 500 sequencing platform (Illumina Inc., San Diego, CA). Mapped reads were generated by TempO-SeqR for the alignment of demultiplexed FASTQ files from the sequencer to the ligated detector oligomer gene sequences using Bowtie, allowing for up to 2 mismatches in the 50-nucleotide target sequence. Counts were assessed using SARTools. Within this R package, edgeR is used for normalization and quality control of count data. Raw/normalized counts and multidimensional scaling between the 4 groups are shown in eFigure 1 (links.lww.com/NXI/A571). Differential expression between groups was assessed by the TempO-SeqR software, which used the DESeq2 method for differential analysis of count data. A significantly differentially expressed gene is defined as having an adjusted p value < 0.05 with no fold-change threshold.
Pathway enrichment analysis was performed using gene sets and pathways defined in the Ingenuity Pathway Analysis software (Qiagen, Germantown, MD) and the ClusterProfiler package\textsuperscript{21,22} in R with default parameters.\textsuperscript{23} ClusterProfiler supports enrichment analysis of Gene Ontology and Kyoto Encyclopedia of Genes and Genomes databases with Gene Set Enrichment Analysis (GSEA) to identify biological themes of a collection of genes. These functional enrichment analyses use computational approaches to identify groups of experimentally observed human genes that are over-represented or depleted in a curated disease or biological function-specific gene set. Additional figures were created using Prism 9 (GraphPad Software, San Diego, CA).

**IHC Analysis**

To confirm sequencing results, 5-μm FFPE sections from 10 GCA/VZV-positive TAs and 10 control/VZV-negative TAs were immunostained for interleukin (IL)-8 and neutrophils (CD15). After deparaffinization, antigens were retrieved in citrate buffer at 95°C for 10 minutes. Slides were immunostained as described\textsuperscript{1} using primary antibodies against IL-8 (mouse anti-IL-8; 1:500 dilution; Abcam, Cambridge, MA; catalog #ab18672) and CD15 (mouse anti-CD15; 1:50 dilution; Abcam; catalog #ab188610). IL-8 and CD15 positive control was normal human tonsil. Slides were imaged by light microscopy.

**Data Availability**

The datasets generated and/or analyzed are available on the NCBI Gene Expression Omnibus database (GSE174694).

**Results**

**Gene Expression Analysis of Control/VZV-Negative and Control/VZV-Positive TAs**

The 31 control TAs were previously immunostained for VZV glycoprotein E (gE) (50 sections from each artery); 20 were negative for VZV gE and 11 were positive for VZV gE (representative staining, Figure 1A). Compared with control/VZV-negative TAs, control/VZV-positive TAs had 78 differentially expressed genes (padj < 0.05; 41 upregulated [red] and 37 downregulated [blue]; Figure 1B; gene list in eTable 2, links.lww.com/NXI/A575). Control/VZV-positive TAs were enriched for pathways involved in viral infections, fibroblast proliferation, and vascular cell migration/spreading (Figure 1C; full gene list in eTable 3, links.lww.com/NXI/A576). For example, the viral infection pathway genes \textit{IFITM2} and \textit{IFITM3} encoding interferon (IFN)-inducible transmembrane proteins that function in viral resistance\textsuperscript{24} were upregulated in control/VZV-positive TAs. Multiple activated (HRAS and ESR1; red) and repressed (MYCN; blue) upstream regulators were identified in control/VZV-positive TAs.
Gene Expression Analysis of Control/VZV-Negative and GCA/VZV-Positive TAs

The 40 GCA TAs were previously analyzed for VZV gE antigen and VZV DNA. All GCA TAs displayed classic biopsy-positive GCA features: transmural inflammation, medial damage, and multinucleated giant/epithelioid cells (Figure 2A, left panel). Twenty TAs contained VZV gE (GCA/VZV-positive) of which 16 were confirmed to have amplifiable VZV DNA (Figure 2A, representative GCA TA with VZV gE). The remaining 20 TAs did not contain VZV gE (GCA/VZV-negative). Compared with control/VZV-negative TAs, GCA/VZV-positive TAs had 1,353 differentially expressed genes (padj < 0.05; 1,095 upregulated [red] and 258 downregulated [blue]; Figure 2B; gene list in eTable 2, links.lww.com/NXI/A575). Top canonical pathways revealed known pathways associated with GCA,25,26 including Th1 and Th2 pathways, CD28 signaling in T-helper cells, T-cell exhaustion, IFN signaling, neuroinflammation signaling, and toll-like receptor (TLR) signaling (Figure 2C). In addition, defined canonical pathways that included virus entry through endocytic pathways were enriched in GCA/VZV-positive samples (Figure 2C; red signifies activation, blue signifies inhibition, and gray signifies unavailable activation pattern). This involved a number of genes encoding endocytic components such as clathrin (CLTA) and the actin-related proteins (ARPC2, ACTR3, and ACTRIB). Multiple cytokines were identified as upstream regulators for GCA/VZV-positive TAs compared with control/VZV-negative TAs, including IFNγ, tumor necrosis factor (TNF), IL-1β, IL-2, IL-15, IL-6, and IL-8 (Figure 2D). In addition, several growth factors (TGFβ1 and KITLG), transcription regulators (STAT1, STAT3, IRF7, and SIRT1), TLRs (TLR3, TLR4, and TLR9), and the transferrin receptor (TFRC) were also linked as key upstream regulators. The major histocompatibility complex class II gene, human leukocyte antigen (HLA)-DRB1, expression was higher in GCA/VZV-positive TAs compared with control/VZV-negative TAs (3.51 ± 1.21; log2Fold-Change ± standard error, p-adj < 0.05), which has been previously reported as a GCA risk factor (reviewed by Carmona et al.25).

Viral Infection and Immune Responses Are Enriched in GCA/VZV-Negative TAs Compared With Control/VZV-Negative TAs

A subset of TAs from patients with GCA were negative for VZV antigen (GCA/VZV-negative). We investigated whether this group is reflective of a nonspecific inflammatory response or potentially represents TAs where detection of VZV antigen (or another virus) was missed because of skip lesions.1 Compared with control/VZV-negative TAs, GCA/VZV-negative TAs had 1,784 differentially expressed genes (padj < 0.05; 1,378 upregulated [red] and 406 downregulated [blue]; Figure 4A; gene list in eTable 2, links.lww.com/NXI/A575). Top canonical pathways of GCA/VZV-negative and GCA/VZV-positive TAs were similar; compared with control/VZV-negative TAs, GCA/VZV-negative TAs were enriched for Th1 and Th2 pathways, CD28 signaling in T-helper cells, T-cell exhaustion, IFN signaling, and neuroinflammation signaling (Figure 4B; red signifies activation, blue signifies inhibition, and gray signifies unavailable activation pattern). As was seen in GCA/VZV-positive TAs, GCA/VZV-negative TAs also showed enrichment for pathways associated with viral infections including nuclear factor kappa B activation by viruses and virus entry through endocytic pathways (Figure 4B). Furthermore, analysis of enrichment scores through GSEA revealed activated gene sets reflective of viral infections, including defense response to virus and TLR signaling (Figure 4C). This included upregulated expression of genes JAK1, JAK2, STAT1, and STAT2 of the JAK-STAT signaling pathway in GCA/VZV-negative TAs. Upstream regulators of differentially expressed genes in GCA/VZV-negative TAs were largely similar to GCA/VZV-positive TAs such as cytokines (IFNγ, TNF, IL-1β, IL-2, IL-15, IL-6, and IL-8), the growth factor TGFβ1, transcription regulators (STAT1, STAT3, and IRF7), TLRs (TLR3 and TLR4), and the iron transporter TFRC (eFigure 3, links.lww.com/NXI/A573; red signifies activation and blue signifies repression).
Predicted Upstream Regulators and Pathways Generated From Unique Differentially Expressed Genes in GCA/VZV-Negative and GCA/VZV-Positive TAs Compared With Control/VZV-Negative TAs

Although GCA/VZV-positive and GCA/VZV-negative TAs displayed similar pathways and upstream regulators compared with control/VZV-negative TAs, we investigated the extent of differentially expressed genes that were shared or unique between GCA TAs without or with VZV antigen compared with control/VZV-negative TAs. GCA/VZV-negative and GCA/VZV-positive TAs shared 1,111 genes that were differentially expressed compared with control/VZV-negative TAs (Figure 5A, middle). Conversely, GCA/VZV-negative vs control/VZV-negative TAs had 673 unique differentially expressed genes that were not found in GCA/VZV-positive vs control/VZV-negative TAs (red signifies activated and blue signifies repressed). GCA = giant cell arteritis; gE = glycoprotein E; IFN = interferon; IL = interleukin; TA = temporal artery; TNF = tumor necrosis factor; VZV = varicella zoster virus.
associated with cell migration and pathologic vascular remodeling in mice.\textsuperscript{28} Enrichment analysis of GCA/VZV-negative unique differentially expressed genes (DEGs) revealed pathways and gene sets associated with cell movement of leukocytes, differentiation of mononuclear leukocytes, replication of Herpesviridae, and viral infection (Figure 5C, left; full gene list in eTable 3, links.lww.com/NXI/A576). Associated pathways and gene sets for GCA/VZV-positive unique DEGs included inflammatory response, peripheral vascular disease, systemic autoimmune syndrome, and viral infection (Figure 5C, right; full gene list in eTable 2, links.lww.com/NXI/A575). Examples of inflammatory genes upregulated included JAK1, TNFRSF10A, NFATC1, and NFKB1.

### Discussion

VZV vasculopathy was initially described as virus infection of cerebral arteries leading to ischemic or hemorrhage stroke, aneurysm, or other cerebrovascular abnormalities that could occur with or without associated rash. Subsequently, VZV vasculopathy expanded to include the extracranial circulation...
with viral antigen found in systemic arteries, including TAs and the aorta. GCA is a systemic vasculitis of the elderly that produces a constellation of symptoms and signs including malaise, headache, TA tenderness, vision loss, elevated C-reactive protein, platelet count, and/or sedimentation rate that can progress to aortitis and stroke; TA biopsies show a transmural vasculitis with medial damage and giant/epithelioid cells. Treatment is with corticosteroids to reduce vascular inflammation; however, the exact cause of GCA is unknown. Previous studies demonstrating that VZV vasculopathy and GCA share clinicohistopathological features and that VZV antigen is found in a large number of GCA TAs suggest that a subset or all of GCA is a form of extracranial VZV vasculopathy. Several other studies did not find VZV antigen in TAs or found it at much lower frequencies; these differences were attributed to nonspecific antigen staining, missed antigen detection because of skip lesions, differences in methodology, and/or insufficient sample sizes analyzed. In addition, it was speculated that if VZV antigen, as well as confirmatory VZV DNA, were present in the GCA TAs, it was merely a bystander and uninvolved in the pathogenesis. Thus, we examined the gene expression profiles of GCA TAs to determine if human transcripts supportive of virus infection were present. Specifically, we completed whole human transcriptome and pathway analysis of GCA TAs with and without VZV antigen (n = 20 in each group) and control TAs with and without VZV antigen (n = 11 and 20, respectively). Our results showed activation of viral and inflammatory pathways in GCA TAs, supporting a pathogenic role for a virus, most likely VZV, in this vasculitis.

The TempO-Seq targeted RNA sequencing assay used in our analysis provides a valuable tool to investigate human transcriptional pathways from archived FFPE tissues without the need for RNA extraction and conversion to complementary DNA; of note, this assay exclusively detects human transcripts, not viral transcripts. The results of TempO-seq analysis of FFPE tissue are comparable with that of fresh and frozen tissue; using RNA samples, TempO-seq generates results, consistent with Affymetrix microarrays and Illumina whole transcriptome RNA-Seq. Furthermore, this assay has been used by other laboratories to identify biomarkers for aggressiveness of clear cell renal cell carcinoma and prognosis in FFPE tumor samples that were then verified by RNA-seq.
Without the limitation of access to fresh or frozen GCA TA tissues, we were able to sequence FFPE samples that have been archived up to 16 years. Importantly, slides can be immunostained for regions of pathology (10 mm², 5–7 μm thickness) then scraped and analyzed. In this study, adjacent sections can be used in IHC to confirm transcriptional pathways, such as the IL-8 and neutrophil infiltration confirmation. Multiple findings of novel human gene expression pathways associated with viral entry and replication and several shared genes in gene sets associated with viral infections, arterial disease, and immune cell activation, supporting a pathogenic role for virus in GCA TAs that contain VZV antigen. Like GCA/VZV-positive TAs, GCA/VZV-negative TAs showed transcriptional signatures associated with viral pathways and robust immune response pathways. These 2 groups were similar, sharing a majority of top canonical pathways. It is possible that individuals with GCA/VZV-negative TAs have cleared viral antigen and now represent a cascade of pathology independent of active viral replication or another pathogen is involved; alternatively, VZV antigen on IHC may have been missed because of skip lesions. Indeed, a comparison of unique transcripts between control and GCA-positive TAs with or without VZV antigen revealed gene sets associated with generalized Herpesviridae infection, potentially representing a contribution of pathogens beyond VZV. Additional epidemiological studies support an association between VZV, and to a lesser extent, herpes simplex virus (HSV), and GCA: (1) among
16,686,345 US participants of whom 5,932 had GCA, complicated and uncomplicated herpetic zoster was associated with a significantly increased risk of GCA (hazard ratios 1.99 and 1.42, respectively, in the Medicare cohort; 2.16 and 1.45, respectively, in the MarketScan cohort)

antiviral treatment was marginally associated with a decreased GCA risk in the Medicare cohort; and (2) among 3,026,005 British Columbia participants of which 4,315 had GCA, the prevalence of GCA in herpes zoster participants (0.34%) was significantly higher than in the general population (0.143%) in the Medicare cohort; and (2) among 3,026,005 British Columbia participants of which 4,315 had GCA, the prevalence of GCA in herpes zoster participants (0.34%) was significantly higher than in the general population (0.143%) among 3,026,005 British Columbia participants of which 4,315 had GCA, the prevalence of GCA in herpes zoster participants (0.34%) was significantly higher than in the general population (0.143%)

the prevalence of GCA in HSV was also higher with the authors concluding that GCA seems to increase with herpetic infections, yet more significantly with zoster.

Aside from detecting novel viral and immune pathways, our results were consistent with previous GCA reports. Specifically, we uncovered pathways associated with Th1 activation, macrophage-mediated tissue disruption through reactive oxygen species, and neutrophil involvement. In addition, TLRs have been implicated in GCA pathogenesis; Deng et al. found that TLR4 activation causes a transmural panarteritis through T-cell recruitment and activation. In this study, TLR4 is a predicted upstream regulator in all GCA TAs. We also found other predicted upstream regulators that have previously been associated with GCA risk or disease severity including IFN-γ, TNF-α, IL-1β, IL-2, IL-6, and IL-10. Finally, multiple studies have shown an association with GCA susceptibility/severity and HLA-class II regions, namely, HLA-DRB1 (reviewed by Carmona et al.) and we also measured higher levels of HLA-DRB1 transcripts in GCA/VZV-positive TAs compared with controls.

Previously, we found that 18% of TAs from normal participants contained VZV antigen with no associated GCA pathology (transmural inflammation, medial disruption, and giant and/or epithelioid cells). The significance of these observations was unclear: was the presence of VZV antigen incidental, not affecting the vascular environment, or because of nonspecific staining with the anti-VZV antibodies used? In our TempO-Seq assay, we found that compared with control/VZV-negative TAs, control/VZV-positive TAs had pathways associated with viral infection, viral replication, budding of virus, and antigen presentation and crosstalk between dendritic cells and natural killer cells, supporting the ability of VZV to still elicit an antiviral response within arterial walls. These participants did not have clinical symptoms, signs, or GCA TA pathology. Yet, the presence of viral pathways raises the possibility that these participants may represent early GCA that may later progress to fulminant disease or that these individuals may have a greater ability to clear virus infection of arteries and/or more appropriately modulate the immune response, preventing aberrant inflammation leading to vasculitis. Importantly, the detection of viral-associated pathways in these TAs without the presence of robust inflammation suggests that the enriched pathways are unlikely nonspecific signatures of infiltrating immune cells and argues against nonspecific VZV antigen staining.

Overall, gene expression and pathway analysis indicated strong evidence for a viral contribution to the pathogenesis of biopsy-confirmed GCA. Although this assay did not specifically identify the virus, among all published viral causes of GCA, VZV is the most biologically plausible, given the compelling clinical-pathological overlap between VZV vasculopathy and GCA. The unbiased finding of viral contributions and host antiviral responses support the immunohistochemical observations of the presence of VZV antigen in GCA TAs (confirmed in many cases by the presence of VZV DNA) and, more specifically, support the ability of virus to induce vascular inflammation that is characteristic of GCA pathogenesis. These findings have the potential to change the standard of care for patients with GCA. Currently, patients with GCA are treated with long-term corticosteroids to reduce the vascular inflammation but not the underlying cause of inflammation; however, steroids have significant side effects particularly in the vulnerable elderly population, such as potentiation of virus infection, accelerated osteoporosis, hyperpertension, hyperglycemia, and irritability. Furthermore, approximately 50% of patients on corticosteroids continue to worsen clinically (vision loss, stroke) or have recurrent disease when steroids are tapered. The addition of an antiviral agent that would treat the underlying cause of vascular inflammation may improve patient outcomes, thus warranting multicenter clinical trials to determine antiviral drug dose and duration, as well as concomitant administration with corticosteroids.

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Disclosure
The authors report no disclosures. Go to Neurology.org/NN for full disclosures.

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Appendix: Authors

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References


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