Rho Kinase Expression in Giant Cell Arteritis Validating pERM Intensity Score as a Method of Increasing Sensitivity of Temporal Artery Biopsy

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Abstract

Background: Aberrant rho-kinase (ROCK) activity is implicated in pathogenesis of several vascular and immunologic disorders. We previously demonstrated evidence of increased ROCK activity in histopathologically negative temporal artery biopsies (TAB) of subjects with clinical Giant Cell Arteritis (GCA) compared to those without GCA. This study aimed to examine ROCK activity in a larger cohort of biopsy-negative GCA subjects and to validate the prior findings.

Methods: Subjects were categorized into 2 groups based on clinical data 6-months after TAB: biopsy-negative GCA and controls without GCA. Paraffin-embedded TAB were stained for phosphorylated ezrin/radixin/moesin (pERM), a surrogate of ROCK activity, and scored by two pathologists blinded to clinical diagnosis using a previously derived scoring system. Three areas of the vessel (intima, adventitial and vasa vasorum) were scored for staining intensity on a scale of 0–2, with a maximum possible score of 6 for each TAB. As determined a priori, scores ³4 were considered a high pERM intensity score correlating with ROCK activity.

TAB sections were also stained for unphosphorylated ERM, the inactive protein.

Results: Thirty six subjects with biopsy-negative GCA and 43 controls were analyzed. There were no differences between groups in age, sex and corticosteroid dose. The mean pERM intensity score in non-GCA subjects was 3.9 ± 1.4 (compared to 5.0 ± 1.4 in those with GCA, p = 0.002). Using the predetermined cut-off of 4 to define high pERM intensity, subjects with GCA were significantly more likely to have a high pERM intensity score compared to non-GCA, OR 3.67, 95%CI :1.19,11.36; p= 0.019. The sensitivity of high pERM intensity score for diagnosis of GCA in histologically negative TAB was 86%, 95%CI: 70,95.

Conclusions:. In this well characterized cohort, those with GCA and negative biopsies had significantly higher pERM intensity scores in TAB specimens compared to subjects without GCA. pERM staining has diagnostic significance in enhancing the sensitivity of TAB, and helps to define the clinically important group of biopsy-negative GCA. The ROCK pathway warrants further investigation in GCA and may be a potential therapeutic target

Background

Giant Cell Arteritis (GCA), the most common systemic vasculitis in adults, is a large vessel vasculitis affecting the aorta and its branches. Many of the classic signs and symptoms of GCA including headache, jaw claudication and vision loss are manifestations of vasculitis and ischemia in the cranial branches originating from the carotid arteries. Because there is risk for ischemic complications in early untreated GCA including permanent vision loss, prompt diagnosis and treatment are essential. As such, temporal artery biopsy (TAB) has long been considered the gold standard to establish a GCA diagnosis. However, TAB is of limited sensitivity rending many false negative results mostly due to non-contiguous inflammation, biopsy technique, delay in obtaining biopsy and lack of temporal artery involvement in a

subset of GCA patients.[1,2] Sensitivity estimates of TAB for the diagnosis of GCA vary widely in the literature with recent data citing a range of 40–70%.[3]

Rho kinases (ROCKs) the downstream target of an intracellular Rho GTPases, help to regulate several critical cellular processes including cytoskeletal organization, motility and gene expression. Through phosphorylation of interferon regulatory factor 4, ROCK is necessary for expression of IL-17 and IL-21, promoting a Th17 immune response. ROCKs also exert activity by activating several components of the actin cytoskeleton, such as ezerin/ radixin/moesin (ERM). In the vascular system, this cytoskeletal regulation promotes smooth muscle contraction and vascular remodeling in response to injury.[4]

Dysregulated ROCK activity is associated with a range of vascular and autoimmune diseases, including hypertension, cardiovascular disease and systemic lupus erythematosus. ROCK activation is implicated in T cell differentiation to Th17 phenotype, inflammatory cell recruitment and vascular remodeling.[5] All of these aberrations are similarly crucial components in GCA pathogenesis, which supports the rationale to explore this pathway in GCA.

Our pilot data showed that ROCK activity was increased in TAB of GCA patients regardless of whether TAB was positive or negative by routine histopathology; thus, suggesting that this pathway may have diagnostic utility in increasing the sensitivity of TAB.[6] The aim of the current study was twofold; firstly, to validate our previously derived pERM intensity score in a larger cohort of biopsy negative GCA patients and secondly, to evaluate role of pERM intensity score in enhancing sensitivity of TAB.

Methods

Patients

TAB obtained as part of the Temporal Artery Biopsy versus Ultrasound in diagnosis of suspected GCA (TABUL) study, an international study comparing TAB and ultrasound in GCA diagnosis were utilized.[7] In TABUL, the treating physician was responsible for assigning a final diagnosis of GCA or no GCA based on clinical data and clinical course at 6 months following presentation. Only histologically negative TABs were used for the current study. Subjects with histologically negative TABs were categorized into two groups based on the TABUL final diagnosis at 6 months: one group was biopsy-negative GCA and the second group was those without GCA who served as controls.

Methods

Paraffin-embedded TAB were sectioned and treated with an immunohistochemical stain for phosphorylated ezrin/radixin/moesin (pERM) (Cell Signaling Technology, Danvers, MA). Each TAB was also stained for unphosphorylated ERM (Abcam, Cambridge, MA), the inactive protein

Antibody standardization and immunohistochemical staining were performed at the Weill Cornell Translational Research Laboratory

Stained slides were reviewed independently by two pathologists blinded to clinical diagnosis. Three areas of the vessel (intima, adventitial and vasa vasorum) were scored for staining intensity on a scale from 0 (no staining) to 2 (high intensity staining) for a maximum possible composite score of 6 in each TAB specimen. As determined a priori, scores ≥4 were considered a high pERM intensity score suggesting increased ROCK activity. Similar scoring was performed for the ERM stained slides.

Each pathologist scored the samples separately. If there was a greater than 2-point discrepancy between the pathologists' composite pERM scores, samples were re-scored. If disagreement was still present, the sample was to be assessed together and consensus score reached.

Outcomes and Statistical Analysis

The primary outcome of interest was pERM intensity score in subjects with biopsy-negative GCA compared to controls (individuals ultimately not diagnosed as having GCA). As determined in our pilot study, pERM intensity was dichotomized into high intensity pERM score (≥ 4) and low pERM score (≤ 3). A similar analysis was performed to compare ERM scores between the GCA subjects and controls. As a secondary outcome analysis of pERM intensity score as a continuous variable (range 0–6) in subjects with GCA compared to controls was performed.

Categorical variables were compared using Fisher's exact test. Continuous variables were tested for normality using the Shapiro-Wilk test. Normally distributed variables were compared using a t-test. Non-normally distributed variables were compared using the Wilcoxon rank-sum test. Statistical significance was defined as $p \leq 0.05$.

A multivariable logistic model was also constructed using stepwise selection methodology. A significance level of 0.10 was required for model entry and a significance level of 0.05 was required to remain in the final model. Diagnosis of GCA was the dependent variable.

Weighted kappa statistics were used to assess inter-rater reliability.

Analyses were conducted using SAS version 9.3 (Cary, NC).

Results

Biopsies of 36 subjects with TAB-negative GCA were identified and compared to biopsies of 43 control subjects without GCA. There were no differences in age, smoking status, diabetes, hypertension, history of myocardial infarction or corticosteroid dosage between GCA subjects and controls (Table 1).

31 of the 36 subjects (86%) with TAB negative GCA had high pERM intensity score compared to 27 of the 43 (63%) controls with high pERM staining, p = 0.01. The mean pERM intensity score in non-GCA subjects was 3.9 ± 1.4 compared to mean pERM intensity score of 5.0 ± 1.4 in those with GCA, p = 0.002. Using the predetermined cut-off of 4 to define high pERM intensity, subjects with GCA were nearly 4 times more likely to have a high pERM intensity score compared to non-GCA, OR 3.67, 95%CI :1.19,11.36; p = 0.019.

(Table 2) In each of the three vascular compartments scored, a significantly higher proportion of GCA subjects had high intensity pERM staining compared to controls without disease. In a multivariate logistic regression, pERM score ≥ 4 remained significantly associated with having GCA, even after adjusting for age and concurrent medications. The sensitivity of high pERM intensity score for the diagnosis of GCA in histologically negative TAB was 86%, 95%CI: 70,95 with a specificity of 63%, 95%CI:47,77.

Nine positive TAB were also stained for pERM. All of these specimens (9/9, or 100%) showed high intensity scores of 5 or 6, in line with previously reported findings.

In GCA subjects, the ERM staining was less intense in TAB specimens compared to the pERM staining, suggesting activation of the ROCK pathway. There was no difference in ERM staining between GCA subjects and controls.

There was a > 2 point discrepancy in pERM intensity score between the two pathologists in only 7 of the 79 biopsies scored for pERM. In only one of these cases did the different scores change the categorization from high pERM score to low pERM score and with repeat scoring there were no > 2point discrepancies necessitating pathologist consensus. There was good inter-rater reliability with a kappa of 0.685 (0.577–0.792).

Discussion

Though still widely performed and considered the gold standard for diagnosis in GCA, TAB has limited sensitivity rendering many false negative results mostly due to non-contiguous inflammation or a large-vessel phenotype. It is widely recognized that even with TAB of sufficient length and appropriate surgical technique, only about 30% of TAB are positive.[8] In untreated GCA, the risk of ischemic complications including irreversible vision loss looms large. As such, the treatment paradigm involves early initiation of high dose glucocorticoids, which themselves are associated with significant potential morbidity in this elderly population. As newer biologic therapies, such as tocilizumab, are introduced for GCA, the onus remains on the clinician to make the accurate diagnosis despite imperfect diagnostic tools. A test, like pERM intensity score, which could enhance the sensitivity of routine histopathology and capture the clinically relevant population of patients with biopsy negative GCA could be of tremendous diagnostic utility. The negative predictive value of high intensity score was substantial at 84%. Such a test could potentially spare patients with negative biopsy from prolonged exposure to glucocorticoids or other immunosuppressive therapy. A strength of this study was confining our analyses to those with negative TAB as prospective clinical data allowed for distinction between those with biopsy negative GCA and those without GCA. Conversely, other promising biomarkers, such as vascular endothelial growth factor (VEGF), that have been looked at in TAB specimens, were only identified in positive biopsies, limiting clinical value.[9]

The current study was limited to subjects with negative TAB. We did not include those with positive TAB in this study as the primary interest was the utility of pERM intensity score in enhancing diagnostic yield

of TAB. We did stain and score nine positive TAB, all of which demonstrated high intensity staining, as expected. As we previously reported, the lymphocytes which infiltrate the artery stain intensely for pERM, making independent analysis of the surrounding vasculature difficult. We also chose to exclude positive TAB in this study as when scoring the negative biopsies, there was true blinding as to clinical diagnosis which is obviously not possible in a positive TAB. One could hypothesize that the inflammatory milieu in early GCA that precedes lymphocyte recruitment causes ROCK activation. Thus, activated ROCK in the vasculature perhaps facilitates the subsequent panarteritis that is pathognomonic of biopsy positivity,

The TAB specimens and clinical data from this study came from the TABUL study, which aimed to compare temporal artery ultrasound to TAB.[7] Using clinical judgement as the reference diagnosis, TAB was reported to have a sensitivity of 39% with 100% specificity compared to ultrasound with sensitivity of 54% and lower specificity of 81%. Variability in sonographer technique and interpretation is often cited as a concern with this user-dependent imaging modality; there was only moderate inter-rater agreement between sonographers who had all undergone a standardized training program. Thus, while there is an understandable desire to use non-invasive imaging techniques to make a GCA diagnosis, at this time ultrasound cannot completely supplant TAB. The use of pERM intensity scoring on a negative biopsy enhanced the sensitivity of TAB for GCA to 86% 95%CI: 70,95..

The findings in the current study serve to validate our previous findings suggesting ROCK activity is increased in the vasculature of subjects with GCA regardless of biopsy status compared to controls. The strong inter-rater reliability between the pathologists scoring the pERM staining further bolster these results. Though we used pERM as a surrogate of ROCK activation in this study, we did also look at the non-phosphorylated ERM protein. There was no difference in the ERM intensity staining in GCA subjects or controls suggesting the pERM differences demonstrated were caused by disease-related activation of the ROCK pathway.

The mechanistic overlap between aberrant ROCK activation and pathophysiologic vascular changes in GCA provide biologic plausibility for these findings. ROCKs play a critical role in maintaining cellular homeostasis and aiding in injury response in the vasculature while simultaneously being necessary for the propagation of the Th17 immune response. In GCA, the simultaneous activation of both the Th1 and the Th17 pathways have long been implicated in disease pathogenesis.[10] The Th17 pathway gives rise to IL-17A expression, which locally enhances the pro-inflammatory milieu and has pleiotropic effects on the surrounding vascular endothelium and smooth muscle. These cell types parallel the areas of high intensity pERM staining and may explain why we can detect pERM staining without an inflammatory infiltrate in those with biopsy negative GCA compared to controls.

While there is immediate diagnostic potential in these current findings if a commercially available stain can improve sensitivity of TAB, there may also be therapeutic implications. Inhibitors of the ROCK pathway have been shown both in vitro and in vivo to reverse T cell dysfunction and downregulate Th17 related cytokines in autoimmune diseases like systemic lupus erythematosus and psoriasis.[11,12] ROCK inhibition likely helps to ameliorate autoimmunity by restoring the balance of regulatory Tcells (Tregs).

Thus, the ROCK pathway may be a viable therapeutic target in GCA. Furthermore, a proposed mechanism for the efficacy of tocilizumab in GCA is that the blockade of IL-6 receptors restores and enhances the suppressive function of Tregs[13] It is conceivable that ROCK inhibition could offer cytokine modulation with similar reestablishment of Treg balance in GCA without the simultaneous immunosuppression seen with IL-6 receptor blockade.

In conclusion, the pERM intensity score can enhance diagnosis of GCA in those with a negative TAB. This underscores the importance of the ROCK pathway in GCA pathogenesis and may offer a promising future therapeutic target.

Declarations

- *Ethics Approval and Consent to Participate*: This study was approved by the Institutional Review Board at Hospital for Special Surgery and Weill Cornell.
- *Consent to Publication:* All authors have reviewed the manuscript and consent to publication.
- *Availability of Supporting Data*: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
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Tables

Table 1
Baseline Demographics for GCA Subjects and Controls

Variable	GCA+ (n = 36)	Controls (n = 43)	p-value
Age (years), mean ± SD	70 ± 10	67 ± 10	0.1898
Smoking, n (%)			0.54
Never/former	32 (89%)	37 (86%)	
Current	4 (11%)	6 (14%)	
Diabetes mellitus, n (%)	4 (11%)	7 (16%)	0.75
Hypertension, n (%)	18 (50%)	25 (58%)	0.50
Myocardial infarction, n (%)	1 (3%)	1 (2%)	1.00
Prednisone dose, median [IQR]	60 [40, 60]	60 [40, 60]	0.49
Aspirin, n (%)	22 (61%)	26 (60%)	1.00
ACE-I/ARB, n (%)	17 (47%)	18 (42%)	0.65
Statin, n (%)	10 (28%)	20 (47%)	0.11

Table 2
pERM Intensity Score in GCA Subjects and Controls

Variable	GCA+ (n = 36)	Controls (n = 43)	p-value
pERM score, median [IQR]	5 [5, 6]	4 [3, 5]	< .001
High pERM score (≥ 4)			0.046
pERM score < 4	6 (16%)	16 (37%)	
pERM score ≥ 4	31 (84%)	27 (63%)	
Intima, n (%)			0.009
0	2 (6%)	2 (5%)	
1	4 (11%)	17 (40%)	
2	30 (83%)	24 (56%)	
Adventitia, n (%)			0.029
0	1 (3%)	5 (12%)	
1	17 (47%)	28 (65%)	
2	18 (50%)	10 (23%)	
Vasa, n (%)			< .001
0	2 (6%)	1 (2%)	
1	6 (17%)	28 (65%)	
2	28 (78%)	14 (33%)	

Figures

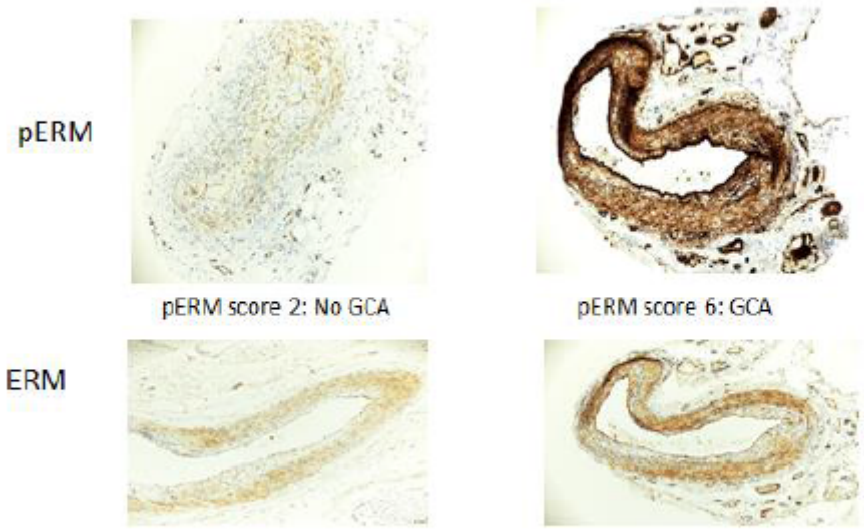

Figure 1

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