Stroke

RESEARCH LETTER

Unveiling Molecular Diversity in Cerebral Thrombi via Spatial Transcriptomics

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Applying spatial transcriptomics (ST) to analyze thrombi represents a novel approach, addressing the challenges posed by their amorphous nature and the difficulty in distinguishing components intrinsic to thrombus versus those from systemic components. Primarily used in oncology, the application of ST in stroke research is relatively unexplored.[1] In this study, we used ST to localize gene expression within thrombi, an approach that offers a distinct advantage over traditional bulk sequencing, which lacks the resolution to detect regional variations within complex tissues. Despite its higher cost, ST allows for detailed mapping of gene expression patterns within discrete regions of interest (ROIs), enabling observation of the molecular dynamics in different parts of a thrombus. ST examines the spatial distribution and molecular composition within their native environment, revealing gene expression patterns and pathways that may differentiate regions prone to causing deleterious effects, such as distal embolization, inflammation, or resistance to thrombolytics. Further development of ST may help identify previously unrecognized therapeutic targets or subtype-specific biomarkers in acute ischemic stroke.

Arterial thrombi from 4 patients with acute ischemic stroke were analyzed, comprising 2 men and 2 women aged 75 to 89 years, with diverse ethnic backgrounds. All subjects received intravenous lytics. Stroke subtypes were determined as large artery atherosclerosis (LAA) or cardioembolic using TOAST (Trial of ORG 10172 in Acute Stroke Treatment) criteria.[2] Thrombi were retrieved during standard thrombectomy from the middle cerebral artery using a triaxial system, ensuring intact retrieval and minimal contamination in a single pass. Samples were formalin-fixed, paraffin-embedded, and sectioned on SuperFrost Plus slides, with adjacent sections stained using hematoxylin and eosin and Giemsa for cluster visualization.[3] For ST, the NanoString GeoMx Digital Spatial Profiling system was used, with 3 fluorescent morphology markers: vimentin for cellular architecture, SYTO-13 (SYTO, Invitrogen, Carlsbad, CA) as a DNA stain, and CD45 (leukocyte common antigen) for hematopoietic cell activation. These markers guided the selection of ROIs based on cellular clustering and morphology. Each ROI underwent UV light exposure to release barcode-tagged oligonucleotides for sequencing. Raw data from the released tags were normalized to the third quartile to correct for variations in cellularity and ROI size. Quality control checks, including sequencing saturation, signal-to-noise ratio evaluations, and spatial autocorrelation analysis, verified uniformity and distribution of gene expression across ROIs[4] (Figure [A and B]).

One of 4 thrombi was excluded from the analysis due to not meeting quality control standards. Twenty-three ROI from 2 LAA and 1 cardioembolic thrombi were available for ST analysis (Figure [A]). To ensure representativeness and coverage of thrombus heterogeneity, each thrombus contributed 7 to 8 ROIs. Among the 18 676 genes analyzed, 4590 were expressed in at least 10% of the ROIs, and 1127 were consistently expressed in at least half of the ROIs. Following third quartile normalization, 8794 genes were identified as actively expressed above the limit of quantitation. The limit of quantitation was determined using the geometric mean of negative probes, adjusted for variability by squaring the geometric SD, ensuring that only genes expressed significantly above background noise were considered (Figure [B]). Differential expression analysis, using linear models, assessed significant variations in gene expression

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across selected ROIs. Each ROI was considered an independent observation, facilitating the investigation of intrathrombus heterogeneity. Linear models were chosen over logistic regression due to the continuous nature of the gene expression data and the small sample size. Volcano plots visualized the differential expression of significantly expressed genes when comparing thrombi (Figure [C and D]). Fold change and significance (*P* value) were calculated based on log2 transformed third quartile normalized counts. Normalized enrichment scores were calculated using Gene Set Enrichment Analysis to highlight the pathways that are the most significantly regulated between specimens. ROIs were compared based on locations and clustering patterns, either in the core or periphery of each thrombus. Of 23 ROIs, 12 were in the core (8 LAA/4 cardioembolic) and 11 in the periphery (7 LAA/4 cardioembolic). Compared with core ROIs in LAA thrombi, those from the periphery were enriched in genes associated with extracellular matrix organization (Figure [E]). Meanwhile, Gene Set Enrichment Analysis revealed more immune response activity in the core of cardioembolic thrombus compared with LAA (Figure [F]).

This exploratory study demonstrates the potential of ST to elucidate complex molecular diversity and intricate expression patterns within cerebral thrombi. Although limited in scope, our findings, differentiated by LAA and cardioembolic stroke subtypes, suggest patterns consistent with the known pathobiology of each subtype.[5] Extracellular matrix enrichment in the periphery of LAA thrombi may represent a barrier of atherosclerotic debris, obstructing lytic therapy and systemic immune processes. Conversely, heightened immune activity in the core of cardioembolic thrombi suggests penetration by systemic or therapeutic processes, potentially making it more effectively targeted. However, these observed differences may also be influenced by patient demographics, comorbidities, and treatment histories and should be interpreted with caution.

ETHICAL APPROVAL

This study was conducted with approval from the University of Washington Institutional Review Board (STUDY00005342).

DATA SHARING

The data that support the findings of this study are available from the corresponding author upon reasonable request. Data will be shared in accordance with institutional and ethical guidelines, ensuring that requests are evaluated on a case-by-case basis to protect patient privacy and comply with data protection regulations.

ARTICLE INFORMATION

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Disclosures

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REFERENCES

1. Bergholtz H, Carter JM, Cesano A, Cheang MCU, Church SE, Divakar P, Fuhrman CA, Goel S, Gong J, Guerriero JL, et al, On Behalf Of The GeoMx Breast Cancer Consortium. Best practices for spatial profiling for breast cancer research with the GeoMx® digital spatial profiler. *Cancers*. 2021;13:4456. doi: 10.3390/cancers13174456
2. Adams HP Jr, Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, Marsh EE 3rd. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. TOAST. Trial of Org 10172 in acute stroke treatment. *Stroke*. 1993;24:35–41. doi: 10.1161/01.str.24.1.35
3. Liu X, Jiang Y, Song D, Zhang L, Xu G, Hou R, Zhang Y, Chen J, Cheng Y, Liu L, et al. Clinical challenges of tissue preparation for spatial transcriptome. *Clin Transl Med*. 2022;12:e669. doi: 10.1002/ctm2.669
4. Zhang L, Chen D, Song D, Liu X, Zhang Y, Xu X, Wang X. Clinical and translational values of spatial transcriptomics. *Signal Transduct Target Ther*. 2022;7:111. doi: 10.1038/s41392-022-00960-w
5. Bingham GC, Lee F, Naba A, Barker TH. Spatial-omics: novel approaches to probe cell heterogeneity and extracellular matrix biology. *Matrix Biol*. 2020;91-92:152–166. doi: 10.1016/j.matbio.2020.04.004

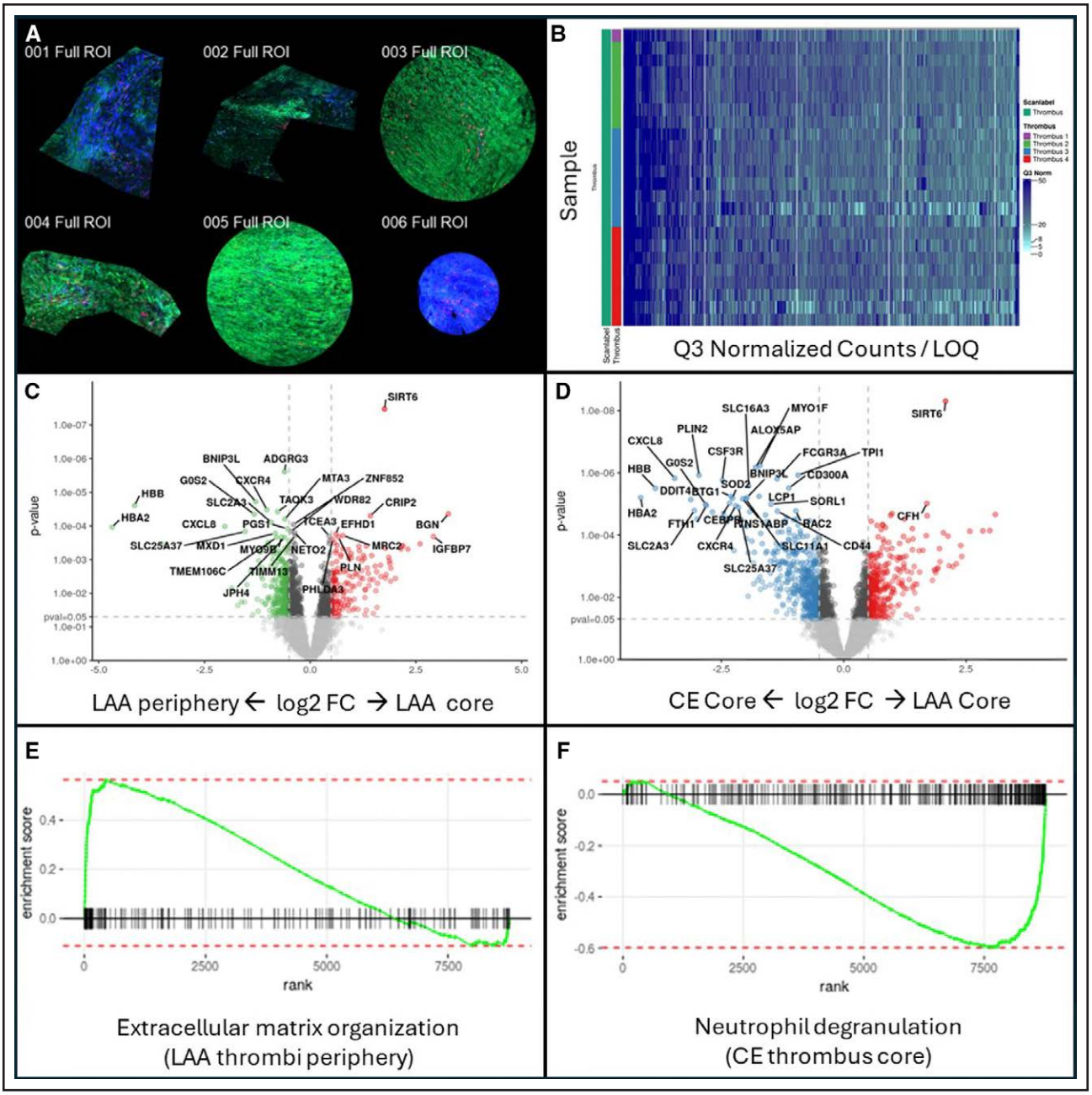

Figure. Spatial transcriptomic analysis of cerebral thrombi.
A, Representative selection from 23 regions of interest (ROIs) using a geometric method based on cellular cluster patterns identified with traditional stains and morphology markers. In **B**, data are sorted by sample type on the *y* axis and show the signal-to-noise ratio, calculated as counts divided by the limit of quantitation (LOQ) on the *x* axis, highlighting the assay's performance across various samples. **C** and **D**, Volcano plots that show differential gene expression, where gene significance is plotted against fold change. Gene symbols are presented according to the standardized nomenclature provided by the HGNC (HUGO Gene Nomenclature Committee). **C** compares 8 large artery atherosclerosis (LAA) core ROIs against 7 LAA periphery ROIs, while **D** compares 4 LAA core ROIs against 4 similarly located cardioembolic (CE) core ROIs. **E** and **F**, Gene Set Enrichment Analysis results. **E**, Significant enrichment of genes associated with extracellular matrix organization in LAA thrombus. **F**, Predominant neutrophil degranulation in the CE thrombus core compared with LAA. In **E** and **F**, the enrichment score is plotted on the *y* axis, which represents the degree to which a gene set is overrepresented at the top or bottom of a ranked list of genes, and the rank is plotted on the *x* axis, indicating the position of genes in the ordered list. A fourth (CE) thrombus did not meet quality control checks and was excluded from the analysis.