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Collagen deposition within brain metastases is associated with leptomeningeal failure after cavity-directed radiosurgery

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Abstract

Background. Leptomeningeal failure (LMF) represents a devastating progression of disease following resection of brain metastases (BrM). We sought to identify a biomarker at time of BrM resection that predicts for LMF using mass spectrometry-based proteomic analysis of resected BrM and to translate this finding with histochemical assays.

Methods. We retrospectively reviewed 39 patients with proteomic data available from resected BrM. We performed an unsupervised analysis with false discovery rate adjustment (FDR) to compare proteomic signature of BrM from patients that developed LMF versus those that did not. Based on proteomic analysis, we applied trichrome stain to a total of 55 patients who specifically underwent resection and adjuvant radiosurgery. We used competing risks regression to assess predictors of LMF.

Results. Of 39 patients with proteomic data, FDR revealed type I collagen-alpha-1 (COL1A1, $P = .045$) was associated with LMF. The degree of trichrome stain in each block correlated with COL1A1 expression ($\beta = 1.849$, $P = .001$). In a cohort of 55 patients, a higher degree of trichrome staining was associated with an increased hazard of LMF in resected BrM (Hazard Ratio 1.58, 95% CI 1.11–2.26, $P = .01$).

Conclusion. The degree of trichrome staining correlated with COL1A1 and portended a higher risk of LMF in patients with resected brain metastases treated with adjuvant radiosurgery. Collagen deposition and degree of fibrosis may be able to serve as a biomarker for LMF.

Key Points

- Collagen deposition and degree of fibrosis may act as a biomarker for LMF.

Importance of the Study

Using proteomic analysis, we identified that a subtype of Type I Collagen is associated with development of leptomeningeal failure in patients with resected brain metastases. We translated this finding into histochemical assay using trichrome as a surrogate for collagen deposition. Given the morbidity and mortality of leptomeningeal disease and its interplay with the tumor microenvironment, the results will be of broad interest to the neuro-oncology community.

Leptomeningeal failure (LMF) is a neurologically devastating variant of cancer progression observed in 5–10% of cancer patients with brain metastases from solid tumors.[1] LMF has been observed in up to 50% of patients after brain metastasis resection and is associated with a rapid decline in quality of life.[2] The median overall survival (OS) of those with untreated LMF is approximately 4–6 weeks and with therapy, 3–6 months.[3,4] The mechanism of LMF development is poorly understood. LMF may arise from intraoperative seeding from a resected parenchymal metastasis,[5] direct spread from extension of a metastasis, or hematogenous dissemination through the blood–brain barrier.[5–10] Recent categorization of LMF has divided the etiology into two distinct radiographic subsets—nodular and classical LMF with differing prognoses and likely different biological underpinnings.[11] Treatments for LMF have variable response rates, and have classically included systemically administered therapy, intrathecal chemotherapy, whole brain radiation (WBRT), stereotactic radiosurgery (SRS),[12] or craniotomy.[1,3]

LMF is more frequently observed after resection compared to SRS for intact brain metastases.[13,14] This finding suggests that surgery may alter the tumor microenvironment and enhance the factors contributing to LMF. Given the high frequency of LMF after resection for brain metastases and its associated impact on survival and quality of life, identification of a biomarker to identify patients at high-risk of developing LMF after resection and adjuvant radiation therapy would represent a significant development. Such a biomarker could aid in the elucidation of mechanisms behind LMF, with subsequent development of molecular targets. Adjuvant therapies that more adequately target the leptomeninges could be employed in high-risk populations.[15,16]

The work in the present study had two sequential aims. First, we sought to identify proteins associated with development of LMF after resection of brain metastases in a heterogeneous group of patients for whom proteomic data was available. Then, we used these proteomic findings to generate a translational hypothesis that could be evaluated with readily available histochemical assays.

Methods

Patient Population

We examined data from patients seen from 2005 to 2016 at a single institution who underwent resection for parenchymal brain metastases from any solid tumor malignancy under an IRB approved protocol (IRB00019774). Patients who had donated brain tissue with metastatic cancer to our institutional tissue bank with adequate clinical follow-up were included. Baseline factors such as age, gender, primary histology, and tumor location were abstracted from the medical record. Patients were managed with resection typically for large, symptomatic brain metastases or for presentation of new, large intracranial masses without histologic confirmation of malignancy. Adjuvant cavity-directed radiosurgery was delivered to the postoperative cavity within 2–6 weeks after resection for the majority of patients, as previously described.[17] Radio-surgical treatment planning was performed using contrast-enhanced, thin-slice magnetic resonance imaging obtained on the day of treatment, unless contraindicated. Patients were generally followed every 3 months with MRI for the first year, then every 4–6 months thereafter.

Clinical Outcomes

Patient outcomes were obtained by review of the electronic medical record. LMF was determined by documentation of clinical records and imaging findings.[11] In brief, LMF was defined as abnormal leptomeningeal enhancement > 5 mm away from the SRS prescription isodose line. Cerebrospinal fluid analysis was not required to confirm LMF. Disease-related enhancement was distinguished from postoperative change by correlating results with the patient's treatment course and defining LMF as > 5 mm away from resection site. LMF was considered to be "nodular" when there was a focus of extra-axial distinct nodular lesion on the meninges or ependyma, or "classical" when MRI findings included sulcal and folial enhancement, linear ependymal enhancement or cranial nerve involvement.[11–18] Of note, 60% of patients had nodular LMF while 40% were of the classical type.

Proteomic Analysis

Proteomic analysis was performed on all available samples within the tumor bank of patients across multiple histology who underwent resection of brain metastases, regardless of adjuvant therapy delivered to the cavity. Prior to analysis, frozen tumor blocks were assessed by a board-certified pathologist (SQ) for adequate, representative tissue. Approximately 20 mg of tissue was lysed in 1 mL of radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitor using a bead mill homogenizer

(Bead Ruptor, Omni International, Kennesaw, GA). RIPA lysate was incubated sequentially with 10 mM dithiothreitol at 55°C for 30 min, and with 30 mM iodoacetamide at room temperature in the dark for 30 min for reducing alkylation of protein. A purified protein pellet was acquired from acetone precipitation, which was then enzymatically digested using sequencing grade modified trypsin. The resulting peptides were desalted using a C18 spin column, dried and resuspended in 5% (v/v) ACN containing 1% (v/v) formic acid for liquid chromatography-tandem mass spectroscopy (LC–MS/MS) analysis.

The LC–MS/MS analysis was performed using a Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Rockford, IL) interfaced with a Dionex Ultimate-3000 nano-UPLC system (Thermo Scientific, Rockford, IL) and a Nanospray Flex Ion Source (Thermo Scientific, Rockford, IL). An Acclaim PepMap 100 (C18, 5 μm, 100 Å, 100 μm × 2 cm) trap column and an Acclaim PepMap RSLC (C18, 2 μm, 100 Å, 75 μm × 15 cm) analytical column were used for the stationary phase. Chromatographic separation was achieved with a linear gradient consisting of mobile phases A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid) where the gradient was from 5% B at 0 min to 40% B at 80 min. MS/MS analysis was performed in data dependent mode for the twenty most intense ions from the full MS scan with dynamic exclusion option for 10 s enabled. Mass spectra were searched with the Sequest HT algorithm within the Proteome Discoverer v2.1 (Thermo Scientific), in combination with the human UniProt protein FASTA database (annotated 20 193 entries, December 2015).

Histochemical Staining

Trichrome histochemical staining was performed on formalin-fixed paraffin-embedded (FFPE) tumor blocks derived from the brain metastasis. The purpose was to translate the results from the proteomic analysis to a readily available assay (trichrome) that may be performed when proteomic analysis is not feasible. The trichrome stain highlights collagenous connective tissue fibers,[19] and is frequently used in staging fibrosis in hepatic cirrhosis.[20,21] Trichrome staining was performed on 5 μm FFPE tumor sections either using the Artisan™ Masson's Trichrome Stain Kit (Dako, Catalogue #AR173) on the Dako ArtisanLink Pro or manually using the Chromotrope 2R with Aniline Blue Trichrome Stain (Poly Scientific R&D Corp; Catalogue # k006). A board-certified pathologist (SO), blinded to the outcomes of the patients and results of proteomic analysis, assessed the degree of trichrome staining specifically within the tumor on a scale of 1–4 for all patients with available tissue blocks with sufficient brain metastasis tumor block remaining.

Statistical Analysis

Patients were stratified by development of leptomeningeal disease. To identify proteins of interest varying between these two groups, we used the empirical Bayes based linear models (limma) and the Benjamini–Hochberg false discovery rate adjustment (FDR). Pearson's correlation was used to assess the relationship between proteins of interest (Type I collagen) and trichrome score of the tissue blocks from the same resected brain metastases.

Translational Cohort

To apply the findings from the proteomic analysis to a scalable, homogeneous group of patients, a total of 55 patients who specifically underwent craniotomy followed by cavity-directed radiosurgery were included in the translational cohort. Fourteen of these patients were from the original proteomic analysis, 41 were from an expanded population of patients for whom proteomic analysis and correlation was not available. Trichrome staining was performed as described above. The cumulative incidence of LMF was estimated by competing risk methodology and compared across strata as described by Fine and Gray.[22] None of these patients in the validation set were included in the proteomic analysis. Tumor blocks from this group of patients were assessed with the same trichrome histochemical assay as detailed above. Sub-distribution hazard ratios (sHR) were estimated to assess the influence of patient and disease factors on the risk of LMF, including degree of trichrome staining as an ordinal variable. Statistical analyses were performed using R (Version 3.3.2; Vienna, Austria).

Results

Proteomic Analysis of Proteins Associated with Leptomeningeal Failure

Thirty-nine patients with proteomic expression data were analyzed. Median follow-up was 12.4 months (95% CI 7.8–26.4 months) after resection of brain metastases. Postoperatively, twenty (52%) received cavity-directed SRS, 8 (21%) received WBRT, 4 were treated with gliadel wafers, 5 patients were observed, 1 received adjuvant radiation at an outside facility, and 1 had intracavitary brachytherapy. Nine (23%) of the resected tumors were recurrent after initial GK or WBRT.

A total of 6407 proteins were analyzed. After FDR correction, collagen type 1-alpha-1 (COL1A1) and collagen type I-alpha-2 (COL1A2) were associated with LMF with statistically meaningful false discovery rates of 0.045 and 0.089, respectively. COL1A1 and COL1A2 exhibited a collinear relationship (adjusted $R^2 = 0.69$). Other proteins of associated with LMF are highlighted in Table 1.

Histochemical Analysis of Samples from Proteomic Analysis

FFPE tumor blocks with sufficient material from the original brain metastasis tissue used for proteomic analysis were available for 27 (69%) of the patients. Because trichrome stains collagenous fibers, trichrome staining was performed on available FFPE blocks to determine if trichrome staining correlated to proteomic expression. The trichrome staining scores were strongly correlated with the

proteomics findings, with the Pearson's correlations of 0.71 (*P*-value = .004) and 0.59 (*P*-value = .026) for the COL1A1 and COL1A2, respectively.

Histochemical Translation of Collagen A1 Association with Leptomeningeal Failure

To translate these findings to a broader context, we sought to determine an association between collagen staining and development of LMD in patients managed for brain metastases. Fifty-five patients who underwent resection followed by cavity-directed radiosurgery for whom original brain metastases tissue was available were analyzed. Patient characteristics are included in competing risks analysis (Table 2). Fifteen patients (36.6%) developed LMD at a median of 7 months after resection. The median overall survival for the entire cohort was 9.7 months (95% confidence interval 5.6–22.2) and median follow-up was 9.4 months (95% CI 5.6–17.2).

The competitive risk analysis ($n = 55$) demonstrated a strong association between trichrome staining score and the risk of LMF (Figure 1). The univariate analysis for the trichrome staining score showed a relative risk of 1.62 (95%

Table 1. Proteins associated with leptomeningeal failure revealed by proteomic analysis of 39 patients with resected brain metastases

LFC*	*P*-value	Symbol	Protein name	Location	FDR**
1.345	.0000473	COL1A1	Collagen type I alpha 1 chain	Extracellular space	0.045
1.202	.000186	COL1A2	Collagen type I alpha 2 chain	Extracellular space	0.089
–0.918	.00436	AKR1B1	Aldo-keto reductase family 1 member B	Cytoplasm	NS
0.952	.00544	KRT19	Keratin 19	Cytoplasm	NS
0.896	.00648	COL18A1	Collagen type XVIII alpha 1 chain	Extracellular space	NS
0.872	.00757	RPS9	Ribosomal protein S9	Cytoplasm	NS
0.85	.00926	PGRMC2	Progesterone receptor membrane component 2	Nucleus	NS
0.871	.0099	SSBP1	Single stranded DNA binding protein 1	Cytoplasm	NS
0.841	.0103	PPP1CA	Protein phosphatase 1 catalytic subunit alpha	Cytoplasm	NS
0.851	.0108	ECI1	Enoyl-CoA delta isomerase 1	Cytoplasm	NS

*Log-fold chain (LFC).
**False Discovery Rate (FDR).

Table 2. Patient characteristics within validation set managed with resection followed by adjuvant cavity-directed radiosurgery

	Total $n = 41$ (Range/percentage)	No LMF* $n = 26$ (Range/percentage)	With LMF* $n = 15$ (Range/percentage)	*P*-value
Location (cerebellar vs other)	10 (24)	5 (19)	5 (33)	.52
Trichrome Score				
1	16 (39)	12 (46)	4 (27)	.078
2	10 (24)	7 (27)	3 (14)	
3	9 (22)	6 (18)	3 (23)	
4	6 (15)	1 (9)	5 (36)	
Male	23 (56)	18 (55)	10 (46)	.71
Age (median)	62 [56–69]	62 [60–69]	60 [51–69]	.36
KPS (median)	80 [70–80]	70 [70–80]	80 [70–80]	.66
Histology				.74
NSCLC*	23 (56)	16 (62)	7 (47)	
Breast	3 (7)	2 (8)	1 (7)	
RCC*	3 (7)	2 (8)	1 (7)	
Melanoma	8 (20)	4 (16)	4 (27)	
Ovarian/GI*	3 (7)	2 (8)	1 (7)	
Small cell lung cancer	1 (2)	0	1 (7)	

*GI, Gastrointestinal, Leptomeningeal failure (LMF); NSCLC, non-small cell lung cancer; RCC, renal cell cancer.

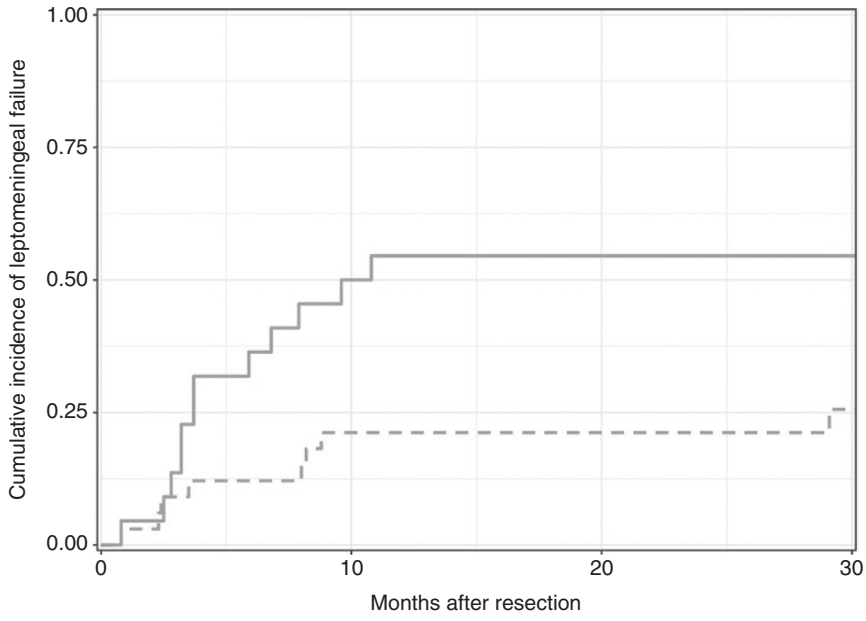

Figure 1. Cumulative incidence of patients who developed LMF after resection and cavity-directed radiosurgery as stratified by trichrome score. Solid line: trichrome score 3–4. Dashed line: trichrome score 1–2. Gray's test *P*-value = .12. (n = 41). .

confidence interval: 1.07–2.44) of statistical significance (*P*-value ≤ .022 of Wald chi-squared test). After adjusting for age and gender, the relative risk of trichrome staining was 1.78 (95% confidence interval: 1.21–2.62, *P*-value ≤ .0037). When stratifying by a trichrome score of 1–3 vs 4, univariate analysis revealed an increased hazard for LMF.

Discussion

The results of this study suggest that the presence of collagen within brain metastases may be associated with a higher risk of LMF following resection and adjuvant radiosurgery. We arrived at these findings by unsupervised FDR adjustment that revealed type I collagen (COL1A1 and COL1A2) in brain metastases was associated with a significantly higher risk of developing LMF. We translated the results of the proteomic analysis to the use of trichrome staining, a simple and affordable histochemistry assay. The degree of trichrome stain correlated closely with the amount of collagen in brain metastasis samples (Figure 2). Additionally, in an expanded dataset, a greater degree of trichrome staining was associated with a higher risk of LMF.

There are several important points that must be noted in interpreting this study. The brain metastases that were analyzed were from specimens obtained prior to the diagnosis of leptomeningeal disease, thus this represents a potential biomarker for developing LMF. Additionally, trichrome is a relatively non-specific stain, but it is commonly used to assess the degree of collagen and connective tissue distribution within cirrhotic livers. Given the stromal profile with multiple connective tissue proteins in addition to type I collagen associated with LMF as highlighted in Table 1, we felt it was reasonable to use a non-specific stain to assess tumor blocks rather than a more costly immunohistochemistry stain specific to COL1A1 and COL1A2.

Our data showed a relatively high percentage of LMF (36%) but this is consistent with other studies of cavity-directed SRS after brain metastasis resection.[23] The cause of this is likely multifactorial. First, many of our patients were treated with adjuvant rather than neoadjuvant SRS, which has been shown to reduce the incidence of leptomeningeal disease.[24] Second, resected brain metastases tend to be larger, with a higher risk of LMF.

This data could influence future therapy by serving as an inexpensive biomarker to evaluate the risk of LMF after resection. The findings could be useful to help guide the clinician to interpret concerning or questionable postoperative imaging. A small indeterminate area of nodular enhancement in a patient with a high trichrome score may warrant further and closer surveillance rather than a patient with a low trichrome score, where postoperative changes or subtle variations in enhancement may skew interpretation. If these findings are replicated in prospective studies with standardized adjuvant treatment, there could be some variation in the treatment paradigm based on trichrome stain or subsequent biomarkers used in LMF-such as WBRT for high-risk patients or targeted therapy with blood–brain barrier penetration.[25,26] Recent data suggests that the use of preoperative radiosurgery may decrease the risk of LMF associated with craniotomy[27], but even with a lower risk, having a biomarker that can risk stratify patients can be useful in the setting of equivocal imaging.

In a broader context, these findings demonstrate the utility of analyzing resected tissue from brain metastases for intracranial patterns of failure. It is common for

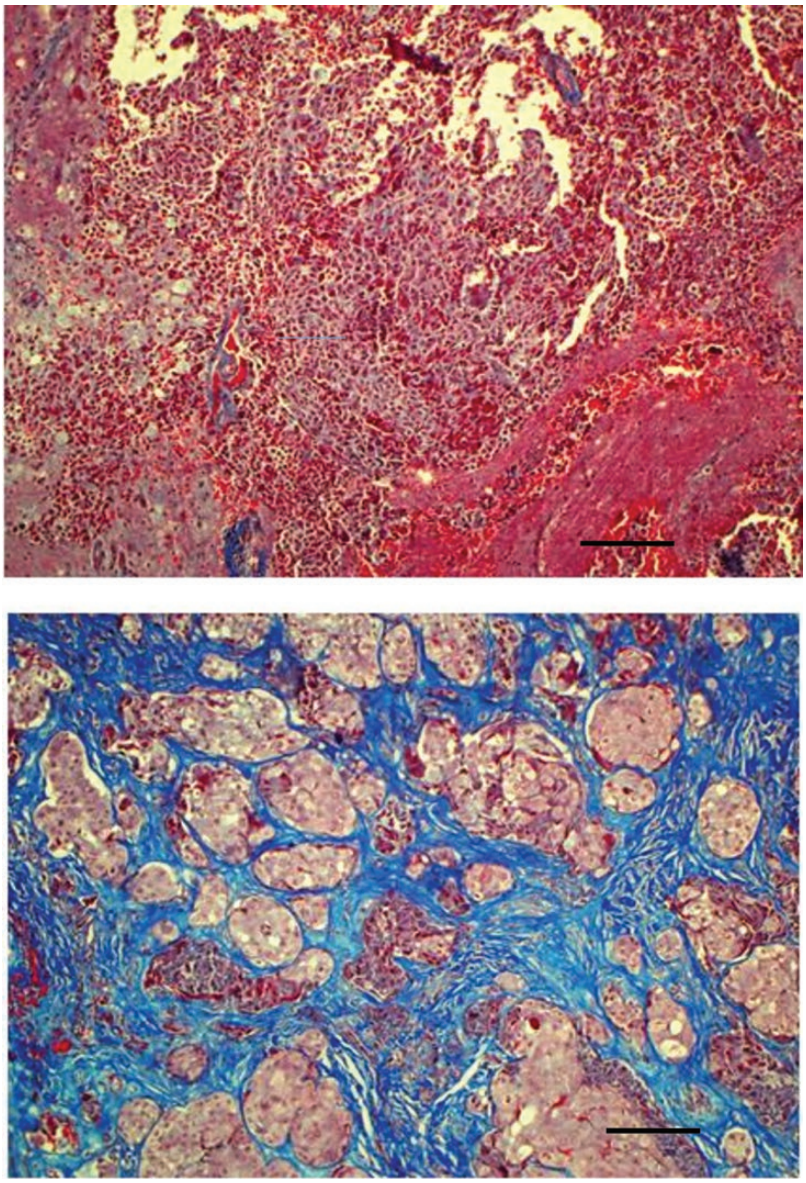

Figure 2. Histologic evaluation of tumors. Metastatic lung adenocarcinoma with minimal collagenous fibrosis (Score 1) in a patient who did not develop LMF stained with trichrome stain (above). Marked collagenous fibrosis (Score 4) in metastatic lung adenocarcinoma in a patient who developed LMF (below). Original objective magnification ×10 (scale bar = 100μm).

resected primary tissue of breast, lung, head and neck, rectal cancer, etc. To be thoroughly examined for high-risk pathologic features such as grade, lympho-vascular space invasion, extra-nodal extension, perineural invasion for prognostic purposes and to guide risk-adapted adjuvant therapies.[28–31] In resection for brain metastases, the surgery serves a more therapeutic rather than diagnostic purpose. It is not standard to evaluate the tumor for predictors of treatment outcomes based on primary tissue analysis. The goal has been fundamentally different between the two treatments—as resection of brain metastases is generally performed for symptomatic purposes or to obtain tissue, preserve neurologic function, and less of an emphasis on clear margins. This is in contrast to oncologic resections of intact primary malignancies where the goal is likely curative and reducing risks of local therapy with or without adjuvant therapy. As patients with brain metastases continue to live longer with improved systemic therapy options, the control of brain metastases and avoidance of devastating progressive disease with LMF is becoming more important.[32] Thus, assessing resected brain metastasis tissue for patterns of intracranial failure should be emphasized and explored. While it could be informative to try to assess collagen deposition in both the primary tumor and the metastatic tissue, this would be beyond the scope of this analysis and infeasible to accomplish given the scarcity of tissue within the stored tumor bank.

Recent advances in biomarker identification for the diagnosis of LMF usually occur when diagnosing pre-existing leptomeningeal dissemination. These advances could ultimately be translated to prediction of LMF. Jiang et al performed next-generation DNA sequencing and identified a population of circulating tumor cells in the peripheral circulation that were lower in the CSF of patients suspected of having leptomeningeal dissemination.[33] Li et al.[34] recently found copy number gains *in MET, ERBB2, KRAS, ALK* and *MYC* in the cell-free DNA of CSF in patients with leptomeningeal dissemination from *EGFR* mutated non-small cell lung cancer. These studies suggest that in isolated populations, the discovery of biomarkers for leptomeningeal dissemination is possible.

With regards to collagen deposition in brain metastases, this was an unexpected finding revealed by unsupervised proteomic analysis. In other tumors, collagen has been implicated as a protein associated with a favorable extracellular matrix within the tumor microenvironment. Collagen fragments degraded in tumor recruit tumor-associated macrophages, resulting in poorer outcomes.[35,36] The additional extracellular matrix from collagen may promote tumor progression by augmenting growth factor signaling and destabilizing cell-cell adhesion.[37] In pancreatic cancer, COL1A1 is associated with disruption of E-cadherin-mediated cell contacts.[38,39] In breast cancer, collagen fibers lining the extracellular matrix have been correlated with poor survival.[40] In mouse models, collagen-1 accumulation is associated in vivo with invasion of the primary tumor, realigning of collagen fibers, and mobilizing of tumor epithelia.[41] Given the role of collagen in promotion of tumorigenesis through extracellular mechanisms, the association between collagen and LMF identified in the current study seems plausible. Further studies are needed to evaluate the role of collagen and collagen formation in resected brain metastases. To our knowledge, this is the first studying investigating collagen in brain metastases.

There are several important limitations of this study, including its retrospective assessment of patient outcomes. Patients who underwent resection generally had large, symptomatic brain metastases, so these findings may not be applicable to smaller brain metastases or brain metastases treated with SRS alone. The adjuvant therapy delivered to patients in this study was heterogeneous: SRS, WBRT, brachytherapy and even observation. Presently, it is unclear whether these modalities can alter the natural history of LMF—though the variability of adjuvant therapy does add a confounding factor to the analysis. Also, systemic treatment delivered to patients prior and after resection of brain metastases is too heterogeneous given the histologic subtypes and multiple lines of therapy to assess. Additionally, the proteomic analysis was performed on a heterogeneous group of patients with regards to adjuvant therapy. Due to financial and logistical constraints, it was not possible to perform proteomic analysis on all 41 patients in the independent dataset. The proteomics data was used primarily to develop a hypothesis that trichrome staining would correlate with risk of LMF and this was tested in a more homogeneous group of patients treated with adjuvant cavity-directed radiosurgery. The relatively small number of patients within each histology likely contributed to lack of statistical power to assess other known risk factors for LMF, i.e. breast cancer, melanoma. Finally, we did not have access to extracranial tissue samples in order to determine whether the presence of collagen was consistent between brain metastasis and extracranial cancer. Given these limitations, this study is limited to hypothesis generation and the findings should be validated prospectively.

Conclusion

After false discovery rate adjustment of protein expression data from brain metastases, type I collagen was associated with development of leptomeningeal disease in patients with resected brain metastases. As a result of this finding, we evaluated the degree of trichrome staining within resected brain metastases. Our results demonstrated that the degree of trichrome staining correlated with risk of LMF in patients with resected brain metastases treated with adjuvant radiosurgery. Trichrome stain could serve as a useful, inexpensive surrogate for expression of stromal components in brain metastases if validated in independent datasets. Thorough histologic evaluation of brain metastases tissue could be used in the future to predict for patterns of failure.

Supplementary material

Supplementary material is available online at *Neuro-Oncology Advances* online.

Supplemental Figure 1. Freedom from leptomeningeal failure in the independent validation cohort ($n = 41$).

Keywords

brain metastases | collagen | leptomeningeal disease | proteomics | trichrome.

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Ethics Approval

IRB00019774.

Consent to Participate

Patients involved in this study signed consent form for IRB approved biorepository.

Consent to Publish

Patients involved in study understood that the data could be used for publication purposes and privacy will be maintained throughout the publication process. The data that support the findings of this study are available on request from the corresponding author, Mohammed Abdulhaleem. The data are not publicly available due to concerns for privacy of the participants in the study.

Authorship Contributions

Experimental design: MA, MS, RH, JJ, MC, JS, KW, LM, WD, JR. Analysis: JS, RH, EM. Implementation: MA, AM, WW, MS, ST, AL, JJ, CF, JL, JR, SO. Interpretation: JS, MS, RH, MC, WW.

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