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CASE REPORT OPEN ACCESS

Epithelial–Mesenchymal Transition and Genetically Driven Early Tumor Seedings in Extraneural Metastasis of Glioblastoma

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ABSTRACT

Genetic and cell biological analyses of this rare glioblastoma (GBM) case with rapidly progressive pleural metastasis revealed essential roles of epithelial–mesenchymal transition (EMT) and glioma stem-like cells (GSCs) in extraneural metastasis. Contrary to the common prediction, the metastatic lesion shared 73% of mutations and two fusion genes with the primary but not the recurrent tumor, indicating that extraneural metastasis occurs early in GBM progression. Functional assays using primary tumor-derived GSCs confirmed EMT-enhanced migratory behaviors. EMT and genetically driven early tumor seedings are crucial for extraneural GBM metastasis, emphasizing the need for further investigation into EMT pathways and GSC biology.

1 | Introduction

Glioblastoma (GBM) is the most common and lethal primary brain tumor, characterized by aggressive invasion of the surrounding brain tissue and inevitable recurrence in the central nervous system. Despite its highly invasive nature, extraneural metastasis is extremely rare, and the specific mechanisms remain unclear. Meanwhile, metastasis is the primary cause of cancer-related death in patients with other types of cancer, and several therapeutic targets for cancer metastasis have been proposed. One plausible theory explaining metastasis involves cancer stem cells (CSCs) through epithelial–mesenchymal transition (EMT) [1, 2]. A single cell is believed to initiate a metastatic lesion, supported by the discovery of CSCs with the capacity for multilineage differentiation and self-renewal. Moreover, the EMT process is supposed to occur opportunistically for CSCs to acquire metastatic ability. CSCs have been identified in GBM [3], as in many types of cancer. However, research has focused on the resistance of glioma stem-like cells (GSCs) to chemotherapy and radiation therapy, which leads to tumor relapse [4]. Regarding the role of EMT in GBM, recent studies identified several EMT-inducing transcription factors linked to GBM progression and invasion into the surrounding brain tissue [5–8]. We recently encountered a patient with GBM who rapidly developed progressive pleural metastasis, which occurred without apparent metastatic triggers. Molecular and genetic analyses of extraneural metastasis of GBM have rarely been reported. In this study, we examined the genetic landscape of this metastatic GBM by whole-exome sequencing and transcriptome analyses and investigated the

Abbreviations: CSCs, cancer stem cells; EMT, epithelial-mesenchymal transition; GBM, glioblastoma; GFAP, glial fibrillary acidic protein; GSCs, glioma stem-like cells; MGMT, O6-methylguanine-DNA methyltransferase; PGSC, primary tumor-derived glioma stem-like cells; RNA-seq, RNA sequencing.

relationship between GSCs and the EMT status and their potential contribution to the extraneural metastasis of GBM using patient-derived CSCs.

2 | Materials and Methods

Detailed information can be found in Data S1.

2.1 | Case Presentation

A 72-year-old man with a history of recurrent chronic subdural hematoma presented with a newly developed right temporal lobe mass during a routine follow-up. Magnetic resonance imaging (MRI) revealed a well-defined intraaxial lesion with ring-like enhancement in the right temporal lobe (Figure 1A). The patient was referred to our institution, and a gross total resection of the tumor was performed. Histopathological examination confirmed glioblastoma, WHO grade IV. Immunohistochemical analysis showed negative staining for $IDH1^{R132H}$ and $BRAF^{V600E}$, while methylation-specific PCR (MSP) revealed an unmethylated O6-alkylguanine DNA alkyltransferase (MGMT) gene promoter. A retrospective histological examination revealed tumor cell infiltration along Virchow-Robin spaces (Figure S1). Postoperatively, the patient underwent conformal radiotherapy (60 Gy in 30 fractions) with concurrent temozolomide therapy, followed by 15 cycles of temozolomide maintenance therapy. Four months after the initial surgery, an MRI revealed a small local recurrence, which was treated with extended-field radiosurgery. However, the lesion continued to grow, necessitating a second surgical resection 2 months later. Histopathological findings confirmed recurrent GBM (Figure 1B). Seventeen months after the initial diagnosis, the patient presented with a right chest pain. Computerized tomography (CT) identified a sizeable pleural mass on the right side (Figure 1C). A biopsy indicated a spindle-cell tumor, initially suggestive of sarcoma, with negative immunostaining for glial fibrillary acidic protein (GFAP) and S-100 (Figure 1C(a)). The pleural lesion progressed rapidly, and the patient ultimately succumbed to respiratory and cardiac failure 19 months after the initial diagnosis. An

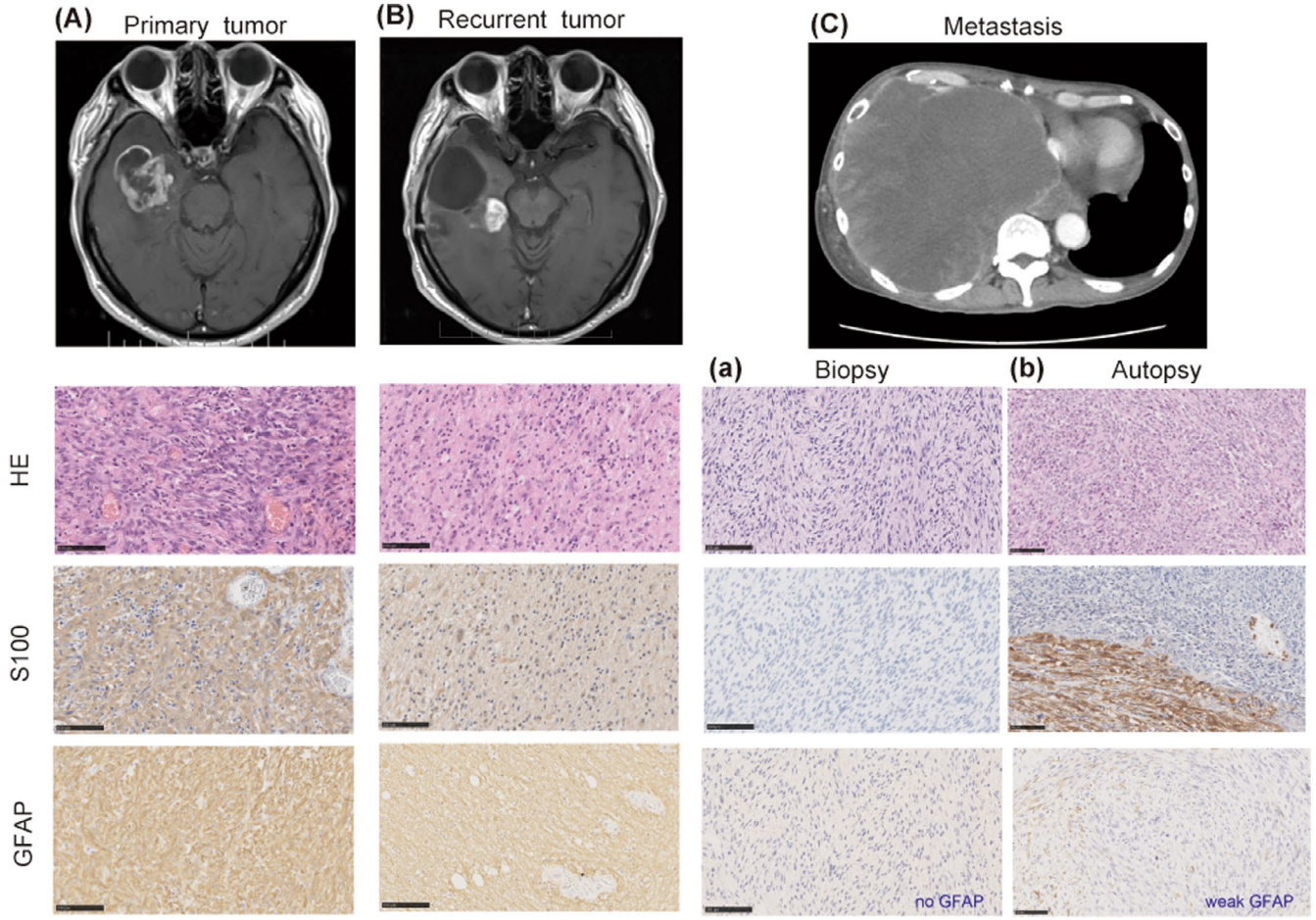

FIGURE 1 | Images and histopathology of the primary tumor, recurrent tumor and extraneural metastasis. Contrast-enhanced MRI revealed the primary (A) and recurrent (B) intracranial glioblastoma lesions. Chest contrast-enhanced CT showed a huge pleural metastasis (C). The correlations of the glioblastoma diagnostic marker expression pattern among the primary tumor, recurrent tumor, and metastatic lesion were assessed by hematoxylin and eosin staining and immunohistochemistry for S100, and GFAP (right; scale bar = 100 μm). The primary and recurrent tumors exhibited strong diffuse immunoreactivity for S100 and GFAP, but the metastasis biopsy (C (a)) was negative for both. The metastatic tumor (C (b)) obtained at autopsy featured strong S100-positive and S100-negative staining areas. Part of the positively stained areas displayed weak immunoreactivity for GFAP.

autopsy revealed the pleural lesion to be an extracranial metastasis of GBM. No further evidence of GBM was found in the brain, spine, or leptomeninges after the second resection for local recurrence. Neither bevacizumab nor steroids were administered during treatment, as the intracranial tumor remained controlled.

3 | Results

3.1 | Genetic Insights into Early Initiation of Extraneural Metastasis Process

Whole-exome sequencing identified 64 shared mutations across the primary, recurrent, and metastatic tumors (Figure 2A). Notably, the metastatic lesion shared approximately 73% of the mutations with the primary tumor, whereas fewer mutations were shared with the recurrent tumor, indicating genetic overlap consistent with metastatic seedings at an early stage. Among the shared mutations, PTEN (P95L) and RB1 (F514fs) were consistently detected in all lesions. PTEN (P95L) is classified as pathogenic in the ClinVar database, while the significance of RB1 (F514fs) remains unknown. TP53 (R333Vfs*12), known to be unique to metastatic tumors, was identified in the metastatic lesion only, suggestive of its possible role in driving the lesion's aggressive behavior. No mutations directly related to EMT were identified (Table S1). RNA sequencing (RNA-seq) identified three fusion transcripts: UHRF1–COL4A2 and DPP9–RAB20 were shared between the primary and metastatic tumors, whereas AHCYL2–CREBZF was detected only in the recurrent tumor, further supporting that the metastatic lesion is genetically closer to the primary lesion than the recurrent lesion. When expression levels of the genes involved in fused transcripts were measured, UHRF1–COL4A2 was associated with significantly higher COL4A2 expression in these lesions compared with other fusion genes (Figure 2B). Additionally, the unmethylated MGMT promoter was detected in all lesions via MSP. RNA-seq revealed that MGMT expression was nearly two-fold higher in the metastatic tumor compared with the primary or recurrent tumors (Figure 2C).

3.2 | EMT and Migration in GSCs

Gene expression profiling analyses revealed that EMT markers, including STAT3, ZEB1, and SNAI2, were highly expressed in primary tumor-derived GSCs (PGSC), the GSCs isolated from the primary tumor of this case (Figure S2). These findings were validated by qRT-PCR demonstrating higher expressions of these transcription factors in PGSC than those of other GSC lines, glioblastoma cell lines, and normal human astrocytes (Figure 2D). Western blotting showed low E-cadherin expression and high levels of mesenchymal markers such as vimentin and β-catenin in PGSC, consistent with mesenchymal characteristics (Figure 2E). Functional assays demonstrated that E-cadherin-negative GSCs, including PGSC, exhibited migratory activity in three-dimensional spheroid migration assays (Figure 2F and Figure S3). PGSC displayed reduced migratory activities compared with other E-cadherin-negative GSCs, consistent with their low CD133 expressions (Table S2). All patient-derived GSCs used in this study show positive expressions of Nestin and Sox2 (Figure S4).

3.3 | Histopathological and Genetic Characteristics of the Metastatic Lesion

Histopathological and protein expression analyses of the metastatic pleural lesion revealed significantly higher expression of E-cadherin (Figure 3A,B), supporting partial epithelial characteristics, and lower expression of mesenchymal markers such as vimentin and β-catenin compared with the primary tumor. Additionally, immunohistochemical analysis showed low GFAP expression in the metastatic lesion, with S-100 positivity observed in certain regions (Figure 1C(b)), indicative of poorly differentiated astrocytic cells undergoing EMT. At the mRNA level, however, expressions of mesenchymal markers, CD44 and S100A1, were lower in the metastatic tumor than in the primary tumor (Figure S5). Nestin was expressed in PGSC regardless of differentiation status (Figure S6).

4 | Discussion

4.1 | Early Extraneural Metastasis Process and Genetic Relatedness

The metastatic tumor showed a higher proportion of shared mutations with the primary tumor than the recurrent tumor and expressed two fusion transcripts common with the primary tumor, contrary to the recurrent tumor expressing a solitary fusion transcript. Elevated COL4A2 expression in the metastatic tumor, likely influenced by UHRF1–COL4A2, highlights its potential role in extracellular matrix remodeling and metastatic competence. This evidence emphasizes the genetic continuity between the primary and metastatic lesions, suggesting the initiation of the extraneural metastasis process and tumor seedings at an early stage of GBM progression.

4.2 | Impact of Temozolomide on Mutagenesis and Progression

Whereas the early initiation of extraneural metastasis was likely driven by inherent genetic factors of the primary tumor, prolonged temozolomide therapy may have further contributed to the selection of aggressive and/or therapy-resistant clones in the metastatic tumor, as evidenced by the emergence of TP53 mutation (R333Vfs*12). The twofold increase in MGMT expression observed in the metastatic tumor aligns with this speculation. These findings underscore the potential mutagenic consequences of extended temozolomide therapy.

4.3 | Role of EMT in Extraneural GBM Metastasis

EMT appears pivotal in GBM extraneural metastases, as EMT-associated transcription factors (STAT3, ZEB1, SNAI2), highly expressed in PGSC, drive mesenchymal characteristics and migratory potential of GSCs. PGSC exhibit reduced migratory activity compared with other E-cadherin-negative GSCs.

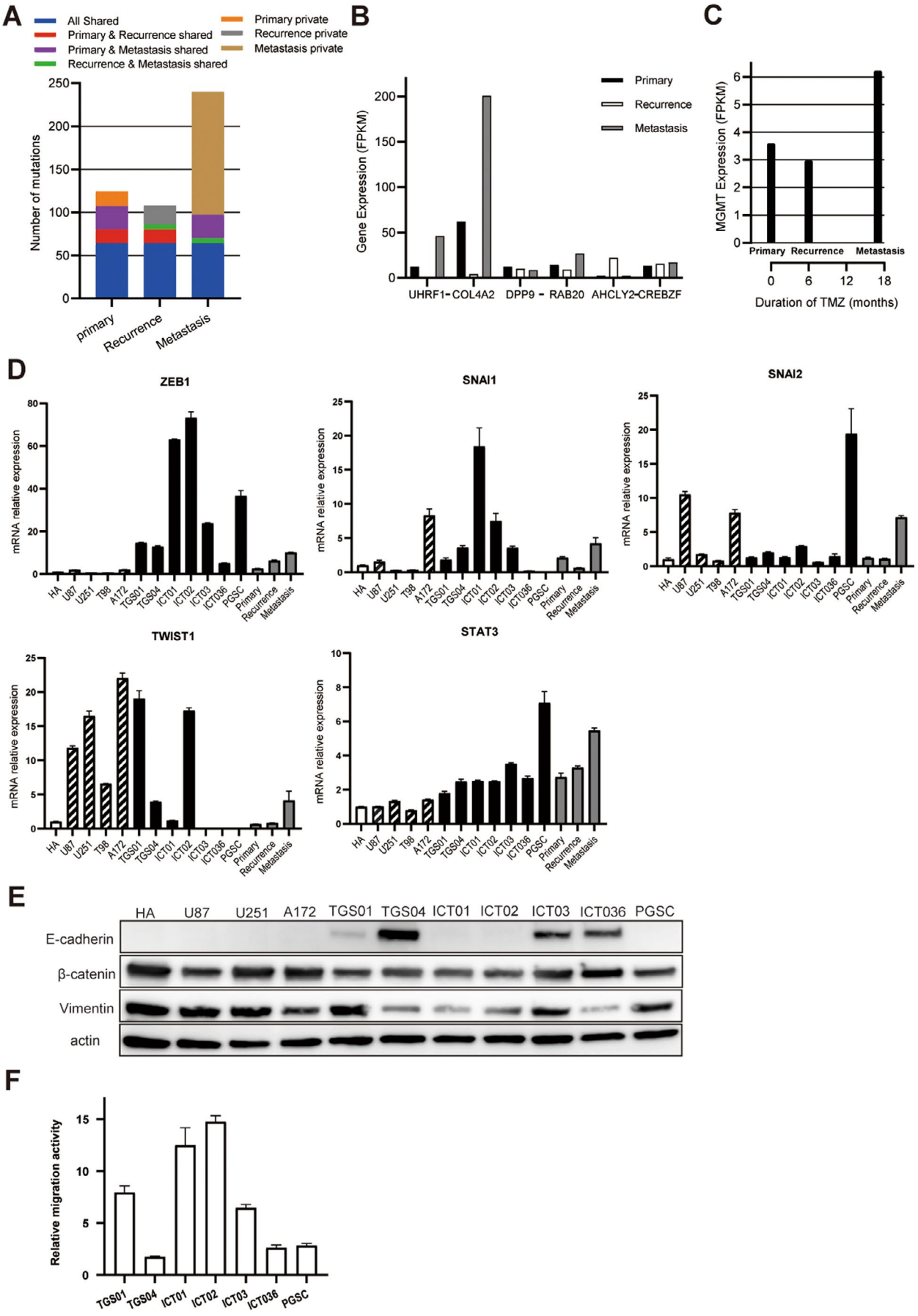

FIGURE 2 | Legend on next page.

FIGURE 2 | Molecular genetics, Western blot, and spheroid migration assay. (A) Mutational burden detected by whole-exome sequencing for the primary, recurrent, and metastatic tumors. (B) Fusion gene transcript expression by RNA-seq. Expression levels (FPKM) of the genes involved in three fusion transcripts are shown for the primary, recurrent, and metastatic tumors. (C) MGMT expression across each tumor as assessed by RNA-seq. (D) qRT-PCR validation of the expression of ZEB1, SNAI1, SNAI2, TWIST1, and STAT3 in various GBM cell lines (U87, U251, T98G, A172) and GSCs (TGS01, TGS04, ICT01, ICT02, ICT03, ICT036), primary tumor-derived GSCs (PGSC) and glioblastoma patient samples. Expression levels were normalized to that of a normal human astrocyte cell line (HA). (E) Patient-derived GSCs were examined by Western blotting to assess E-cadherin, vimentin, and β-catenin protein expression in comparison with GBM cell lines and HA. (F) Migration of GSCs in a spheroid migration assay. The area covered by migrated cells was quantified after 72 h and presented relative to the initial sphere at 0 h. The bar graph presents the mean ± SEM of triplicates.

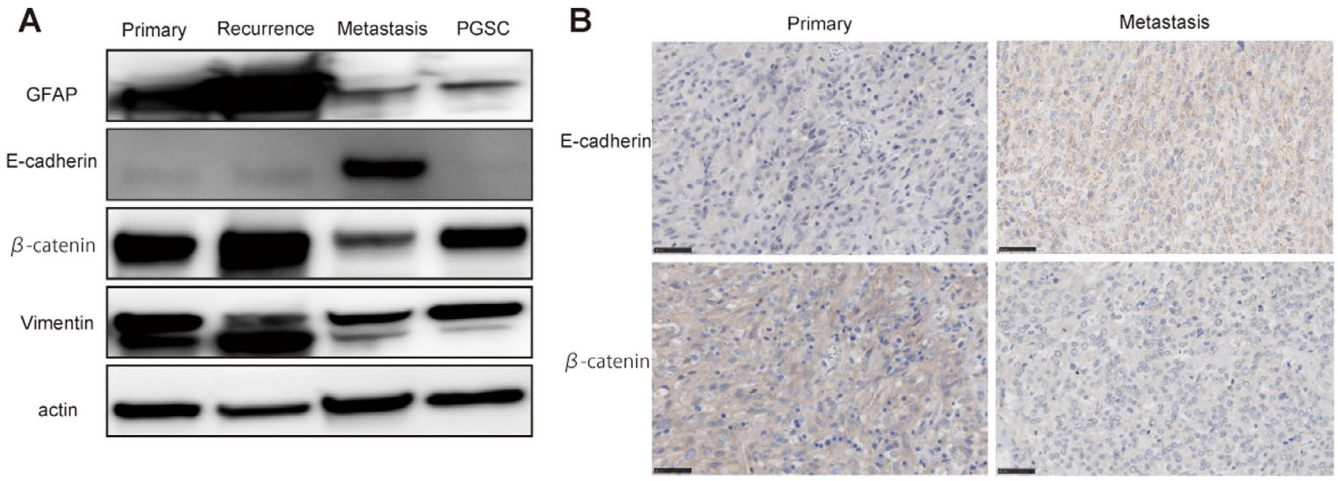

FIGURE 3 | Epithelial-mesenchymal transition changes in the extraneural metastasis. (A) EMT changes in the metastatic GBM lesions were examined by Western blotting. The protein expressions of vimentin and β-catenin were decreased in the metastasis compared with those in the intracranial GBM samples, whereas E-cadherin expression was significantly increased. GFAP expression was significantly lower in the metastasis and in the primary tumor-derived GSCs (PGSC). (B) Immunohistochemical examination of the metastatic glioblastoma lesion. E-cadherin expression was high in metastatic tumor cells but absent in primary tumor cells. Unlike E-cadherin, β-catenin exhibited high cytoplasmic expression in primary tumor cells but not in metastatic tumor cells (scale bar = 50 μm).

The reduced migratory capacity is likely related to low CD133 expression, a marker associated with stem cell properties. Differences in CD133 expression and migratory abilities among GSC populations may influence tumor metastasis capabilities. In addition, the low GFAP expression observed in the pleural metastatic lesion suggests the involvement of EMT, further supporting its role in the early initiation of extraneural metastasis. On the other hand, histopathological and molecular analyses of the pleural lesion also revealed partial epithelial characteristics, with elevated E-cadherin expression and reduced mesenchymal marker expression (e.g., vimentin and β-catenin). Whereas EMT is crucial for GBM to initiate the extraneural metastasis process, mesenchymal-epithelial transition may further occur after metastasis, facilitating colonization and growth outside the central nervous system.

Author Contributions

Seisaku Kanayama: conceptualization, data curation, investigation, methodology, writing – original draft. **Kentaro Watanabe:** data curation, formal analysis, investigation. **Minoru Tanaka:** writing – review and editing. **Junko Takita:** data curation, formal analysis, investigation. **Tomoki Todo:** conceptualization, funding acquisition, investigation, methodology, supervision, writing – review and editing.

Ethics Statement

Patient tumor samples and clinical data were obtained for use in future research. Written informed consent was provided under a protocol reviewed and approved by the Institutional Review Board of the Institute of Medical Science, the University of Tokyo. Written consent was obtained from the patient for publication. Registry and the Registration, N/A. Animal Studies, N/A.

Conflicts of Interest

Tomoki Todo and Junko Takita are editorial board members of Cancer Science. The other authors have no conflicts of interest to declare.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.