ORIGINAL ARTICLE

WILEY Cancer Science

High G2 and S-phase expressed 1 expression promotes acral melanoma progression and correlates with poor clinical prognosis

Tianxiao Xu | Meng Ma | Zhihong Chi | Lu Si | Xinan Sheng | Chuanliang Cui |
Jie Dai | Sifan Yu | Junya Yan | Huan Yu | Xiaowen Wu | Huan Tang | Jiayi Yu |
Yan Kong (iD) | Jun Guo

Key Laboratory of Carcinogenesis and
Translational Research (Ministry of
Education/Beijing), Department of Renal
Cancer and Melanoma, Peking University
Cancer Hospital & Institute, Beijing, China

Correspondence
Jun Guo and Yan Kong, Department of
Renal Cancer and Melanoma, Peking
University Cancer Hospital & Institute,
Beijing, China.
Emails: guoj307@126.com and
k-yan08@163.com

G2 and S-phase expressed 1 (GTSE1) regulates cell cycle progression in human cancers. However, its significance and mechanism of action in acral melanoma (AM) remain unknown. In the present study, we found that GTSE1 expression was upregulated in advanced stage/metastatic AM tissues and metastatic cell lines, and correlated with higher stage ($P = .028$) and poor disease-free survival (DFS) in patients with AM ($P = .003$). Cox regression assays validated GTSE1 expression to be an independent prognostic factor of DFS for patients with AM ($P = .004$). Ectopic expression of GTSE1 enhanced primary AM cell proliferation, invasion, and migration. Loss-of-function in GTSE1 attenuated metastatic AM cell proliferation and metastatic ability in vitro and in vivo. We additionally observed that inhibition of migration and invasion occurred concomitantly with a GTSE1 knockdown-mediated increase in E-cadherin and decreases in N-cadherin and Slug. We further showed that integrin subunit alpha 2 (ITGA2) interacts with GTSE1 and is a downstream effector of GTSE1. Further, ITGA2 levels were positively correlated with GTSE1 expression in human AM tissues. Ectopic ITGA2 expression rescued siGTSE1-mediated inhibition of migration and invasion, thereby restoring epithelial-to-mesenchymal transition (EMT). In conclusion, GTSE1 expression promotes AM progression and correlates with clinical outcomes of patients with AM, and may represent a promising therapeutic target to suppress AM progression.

KEYWORDS
acral melanoma, epithelial-mesenchymal transition, G2 and S-phase expressed 1, integrin subunit alpha 2, metastasis

1 | INTRODUCTION

Acral melanoma is the predominant melanoma subtype in non-Caucasians,[1-3] particularly in China, accounting for almost 50% of all melanomas.[4] Compared with common cutaneous melanoma, acral

melanoma (AM) has a poorer prognosis[2,3] and shows a markedly different genomic landscape, with a far lower mutation burden dominated by larger-scale genomic rearrangements.[5,6] In patients harboring BRAF and c-KIT mutations, vemurafenib and imatinib elicit greater therapeutic effects than traditional chemotherapies.[7-10] However, the mutational frequency of BRAF and c-KIT is approximately only 16%[11,12] and 12%[12-14] in AM, respectively, meaning that most patients with AM are ineligible for treatment with current targeted therapies. Therefore, identification of additional therapeutic targets for patients with AM is imperative.

Distant metastasis is a significant cause of mortality in melanoma;[15] however, to date, no efficient therapeutic strategy tackling the metastatic properties of melanoma is clinically available. Recent studies have largely focused on the development of targeted therapies and immunotherapies with fewer reports of therapeutic strategies aimed at suppressing invasiveness.[16] However, patient resistance to targeted treatment and insensitivity to immunotherapy is often linked to invasiveness.[17,18] Therefore, an improved understanding of the mechanisms underlying metastasis, and the identification of novel therapeutic targets for AM will should enable advances in the treatment of this rare but aggressive melanoma subtype.

G2 and S-phase expressed 1 (GTSE1) has been found expressed only in the S and G2 phases of the cell cycle, where it colocalized with tubulin and microtubules,[19,20] and is overexpressed in lung cancer,[21] breast cancer,[22,23] and liver cancer.[24] Accumulating evidence indicates that GTSE1 correlates with tumor metastasis and poor clinical outcome in neuroblastoma,[25] neuroendocrine tumors,[26] oral tongue squamous cell carcinoma,[27] and hepatocellular carcinoma.[24] In response to DNA damage, GTSE1 accumulates in the nucleus, where it downregulates p53 and represses its ability to induce apoptosis.[28-30] Negative regulation of p53 allows cells to resist apoptosis and transition through the G2/M checkpoint, leading to tumor progression. In interphase, GTSE1 accumulates at growing microtubule plus-ends by interacting with microtubule-associated protein RP/EB family member 1 (EB1), and its activity is required for cell migration and focal adhesion formation.[31] GTSE1 has been found to play roles in human cancer proliferation, apoptosis, and migration.

In the present study, we confirmed the increased expression of GTSE1 in metastatic AM and showed its correlation with clinical outcome. GTSE1 was shown to play an important role in AM progression, regulating AM migration and invasion by disrupting epithelial-to-mesenchymal transition (EMT). Our findings indicate that GTSE1 represents a promising therapeutic target for AM.

2 | MATERIALS AND METHODS

2.1 | Patients and specimens

Six pairs of primary AM tissues and corresponding lymph node metastases were recruited for western blots. Each specimen was immediately snap-frozen and stored at −80°C. Another group of primary paraffin-embedded AM samples from 92 patients and metastatic paraffin-embedded AM samples from 45 patients (including 11 subcutaneous metastases, 1 intestinal metastasis, 1 lung metastasis, and 32 lymph node metastases) were recruited for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and immunohistochemistry (IHC). All the samples were analyzed by H&E staining and by IHC to confirm the diagnosis of melanoma at Peking University Cancer Hospital & Institute from 2013 to 2016. Medical records of the patients were reviewed to collect information on age, gender, tumor thickness (Breslow), ulceration, TNM stage, and disease-free survival (DFS). This study was approved by the Medical Ethics Committee of the Peking University Cancer Hospital & Institute and was conducted according to the principles of the Declaration of Helsinki. Informed consent for use of material in medical research (including archiving materials and establishment of cell lines) was obtained from all participants.

2.2 | Cell lines and primary cell culture

As previously described,[32] an AM cell line-1 (AMC-1 cell line) and a matched metastatic AM cell line-2 (AMC-2 cell line) were derived from a hospitalized patient with AM. The WM115 cell line was derived from the primary tumor of a malignant melanoma, and the WM2664 cell line was derived from a metastatic site in the same patient. The A2058 cell line was derived from a lymph node metastasis of melanoma. These 3 cell lines were obtained from the ATCC (catalog numbers: CRL-1675, CRL-1676, and CRL-11147, respectively). Cells were cultured at 37°C in DMEM (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) supplemented with penicillin and streptomycin (Invitrogen) and containing 10% FBS (HyClone; GE Healthcare, Logan, UT, USA).

2.3 | Quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted by lysing cells and formalin-fixed, paraffin-embedded sections with a GeneJET RNA Purification kit and a RecoverAll™ Total Nucleic Acid Isolation Kit (both from Thermo Fisher Scientific), respectively. Total RNA was converted to first-strand cDNA using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), and measured by qRT-PCR using the Applied Biosystems® 7500 Fast Real-Time PCR System and SYBR Green, according to the manufacturer's instructions (Thermo Fisher Scientific). GAPDH was used as an internal control. Primer sequences are shown in Table S1. Human epidermal melanocyte (HEM)-light, HEM-medium, and HEM-dark RNA was purchased from ScienCell Research Laboratories (Santiago, CA, USA). We used mixed HEM RNA as a control. The $2^{-\Delta\Delta CT}$ method was used to determine relative gene expression levels, and each experiment was repeated at least 3 times.

2.4 | Immunohistochemistry

Immunohistochemistry was carried out on paraffin-embedded tissue samples using antibodies against GTSE1 (1:400; Proteintech,

Chicago, IL, USA) and integrin subunit alpha 2 (ITGA2) (1:400; Abcam, Cambridge, UK), followed by a standard avidin-biotin detection protocol using 3-amino-9 ethylcarbazole. Sample staining was independently scored as 0 (negative), 1, 2, or 3 (weak, intermediate, and strong, respectively) by 3 pathologists, based on both the proportion of positively stained tumor cells and their staining intensity. GTSE1 protein expression was classified as high (IHC score of 2 or 3) or low (IHC score of 0 or 1) expression.

2.5 | Transfection

Small interfering RNAs (siRNAs) targeting GTSE1 were designed and synthesized by RiboBio (Guangzhou, China). The sequences are shown in Table S1. A non-targeting siRNA (siCTL) was used as a negative control. Cells were transfected using Lipofectamine RNAi-MAX (Invitrogen, Shanghai, China) mixed with 10 nmol/L siRNA.

FLAG-tagged-pCMV3-GTSE1, pCMV3-ITGA2, FLAG-tagged-pCMV3-control and pCMV3-control expression plasmids were purchased from Sino Biological (China). Transient transfection of plasmids into melanoma cells was done using Lipofectamine 3000 (Invitrogen, Shanghai, China) following the manufacturer's protocol.

For stable transfection, a lentiviral vector overexpressing GTSE1 (pEZ-Lv201) and a red firefly luciferase cloning vector with shRNA against human GTSE1 (psi-LvRU6rLP) were designed and synthesized by GeneCopoeia (Guangzhou, China). The sequence is shown in Table S1. Lentiviruses were mixed with Polybrene (5 mg/mL) and added to melanoma cells. Positive clones were selected in puromycin (5 mg/mL). Stable transfectants were isolated after 2 weeks.

2.6 | In vivo tumorigenicity assay

All animal experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals with protocols approved by the Animal Care and Use Committee at Peking University Cancer Hospital & Institute.

For the tumorigenicity assay, AMC-1-control and AMC-1-GTSE1 cell suspensions (2×10^6 cells/mouse) in PBS were s.c. injected into 6-week-old non-obese diabetic-SCID (NOD-SCID; Vital River, Beijing, China) female mice. Twelve mice for each cell line were divided into 2 groups: mice injected with control cells (n = 6) and mice injected with GTSE1 cells (n = 6). Tumor volume was determined using the formula $V = L \times W^2 \times 0.5$, where L and W represent the largest and the smallest diameters, respectively. Tumor formation was monitored weekly.

2.7 | Metastatic mouse model and luciferase detection

We injected AMC-2-shCTL or AMC-2-shGTSE1 cells (1×10^6 cells/mouse) into 6-week-old female NOD-SCID mice by the tail vein. After 30 days, mice were injected ip, with D-luciferin (Promega, Madison, WI, USA) and anesthetized with 1%-3% isoflurane. Bioluminescence intensity (BLI) measurements, expressed as photons per

second (p/s), were carried out to evaluate the stably luciferase-transfected AMC-2 cells, using a highly sensitive cooled charge-coupled device camera mounted in a light-tight specimen box (IVIS; Xenogen, Waltham, MA, USA). Finally, organ tissues were fixed in formalin, embedded in paraffin, sectioned, and stained with H&E.

2.8 | Immunoprecipitation

Cells were harvested in Nonidet P-40 lysis buffer containing the protease inhibitor PMSF (1:100). To preclear the samples, protein A/G agarose (Beyotime Biotechnology, China) and mouse/rabbit IgG antibodies were added to the lysates and incubated for 2 hours at 4°C. Immunoprecipitation (IP) experiments were carried out in the same buffer with either mouse monoclonal Flag antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit monoclonal ITGA2 antibodies (Abcam) and protein A/G agarose and incubated overnight at 4°C. After this incubation, the samples were centrifuged, and the beads were washed with NP-40 lysis buffer 3 times. The remaining beads were denatured at 99°C for 10 minutes. Then, cell lysates and immunoprecipitates were separated by SDS-PAGE and subjected to western blot analysis with FLAG, GTSE1 (mouse polyclonal; Abcam), and ITGA2 antibodies.

2.9 | Statistical analysis

SPSS 16.0 software was used for all statistical analyses. Data represent the mean $\pm$ SD. We used Student's t test, chi-squared test, and Fisher's exact test to evaluate differences between 2 groups. DFS was defined as the interval from the initial surgery to the point of clinically defined recurrence or metastasis. Postoperative DFS probability was determined by the Kaplan-Meier method, and log-rank tests were used to estimate statistical significance between the time-dependent outcomes of DFS. Hazard ratios and confidence intervals at the 95% level were determined using multivariate Cox regression analysis. All statistical analyses were 2-sided, and $P < .05$ was considered statistically significant.

Detailed description of western blots, cell proliferation assays and cell cycle analysis, as well as wound healing, transwell and invasion, and transcriptome profiling analysis are provided in Data S1.

3 | RESULTS

3.1 | G2 and S-phase expressed 1 is upregulated in metastatic AM tissues and cell lines

To determine GTSE1 expression in metastatic AM in situ, GTSE1 mRNA and protein levels were evaluated in 137 human samples (92 primary sites and 45 metastases). GTSE1 mRNA expression was significantly upregulated in metastatic tissues compared to primary tissues ($P < .001$; Figure 1A). The high expression of GTSE1 in metastatic tissues in situ was further confirmed by IHC. GTSE1 protein expression was significantly upregulated in metastatic tissues ($P = .016$; Figure 1B). To examine GTSE1 expression in paired AM

tissues, 6 pairs of primary and metastatic AM tissues were observed. As shown in Figure 1C, GTSE1 protein levels were significantly elevated in metastatic AM tissues compared with matched primary tissues. We also analyzed GTSE1 mRNA and protein levels in melanoma cell lines. Compared with primary AMC-1 and WM115 cells, matched highly metastatic AMC-2 and WM2664 cells showed elevated mRNA and protein levels (Figure 1D,E). Taken together, the results indicate that GTSE1 expression is increased in metastatic AM tissues, implying that GTSE1 upregulation may lead to AM progression.

3.2 | High GTSE1 expression predicts poor prognosis for AM patients

To address the relationship between GTSE1 expression and clinical outcome, 92 primary AM patients were recruited. Based on the GTSE1 staining scores of the resected tumors, 48 patients were categorized as having high expression, and 44 as having low expression. Patients and their clinical characteristics are summarized in Figure 2A and Table S2. There were no statistically significant differences between the groups with respect to age, gender, tumor thickness, or ulceration. Patients with higher GTSE1 expression tended to have advanced stage AM ($P = .028$, Figure 2B). Kaplan-Meier analysis showed that patients with high GTSE1 expression had a strikingly higher incidence of recurrence and metastasis, and high GTSE1 expression was strongly correlated with short DFS compared with GTSE1 low expression (11.1 vs 16.1 months, $P = .003$; Figure 2C), indicating that GTSE1 may be useful in predicting the clinical prognosis of AM. Univariate analysis showed that TNM stage, thickness, and GTSE1 protein expression were predictive of poor outcomes. In Cox multivariate analysis, GTSE1 protein expression ($P = .004$) and clinical staging ($P < .001$) were the strongest individual clinical factors (Table 1). Taken together, the data suggest that GTSE1 represents a useful prognostic biomarker for patients with AM.
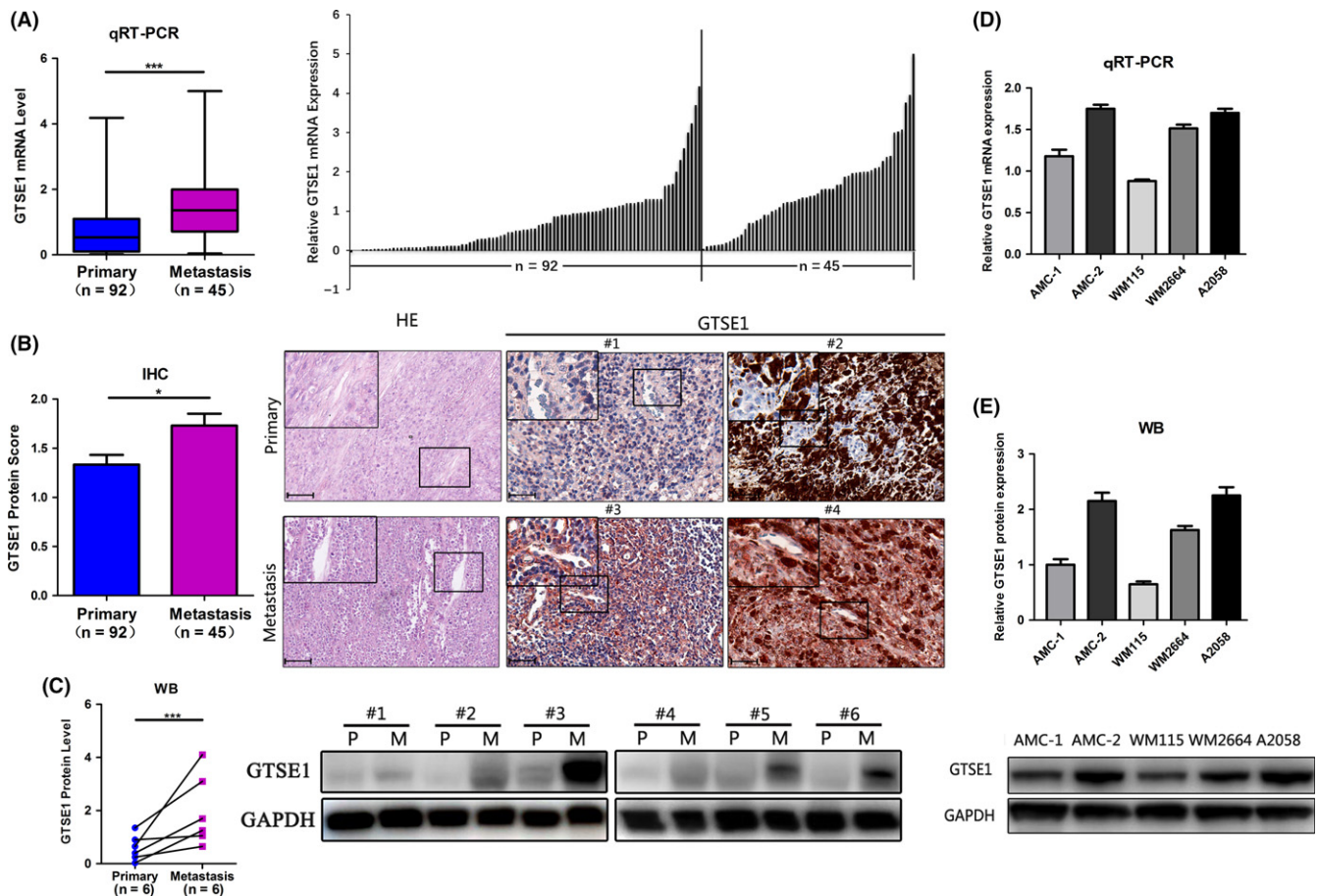
FIGURE 1 Upregulation of G2 and S-phase expressed 1 (GTSE1) expression is common in metastatic acral melanoma (AM) tissues and cells. A, qRT-PCR analysis of GTSE1 mRNA levels in tissues of patients with AM. B, Representative examples of GTSE1 immunohistochemistry (IHC) staining in primary and metastatic AM tissues are shown. Statistical analysis of GTSE1 scores is shown in the left panel. Scale bars, 500 μm. Magnification, 40×. C, Western blot (WB) analysis was carried out to determine GTSE1 protein levels in 6 primary AM tissues and their corresponding metastatic tissues. The left panel shows quantification of the western blot data; the right panel shows representative images of GTSE1 expression in primary and adjacent metastatic AM tissues. D,E, mRNA and protein levels of GTSE1 were determined in 5 melanoma cell lines by qRT-PCR and western blotting. GAPDH was used as an internal control. *$P < .05$, ***$P < .001$

(A)

(B)

(C)
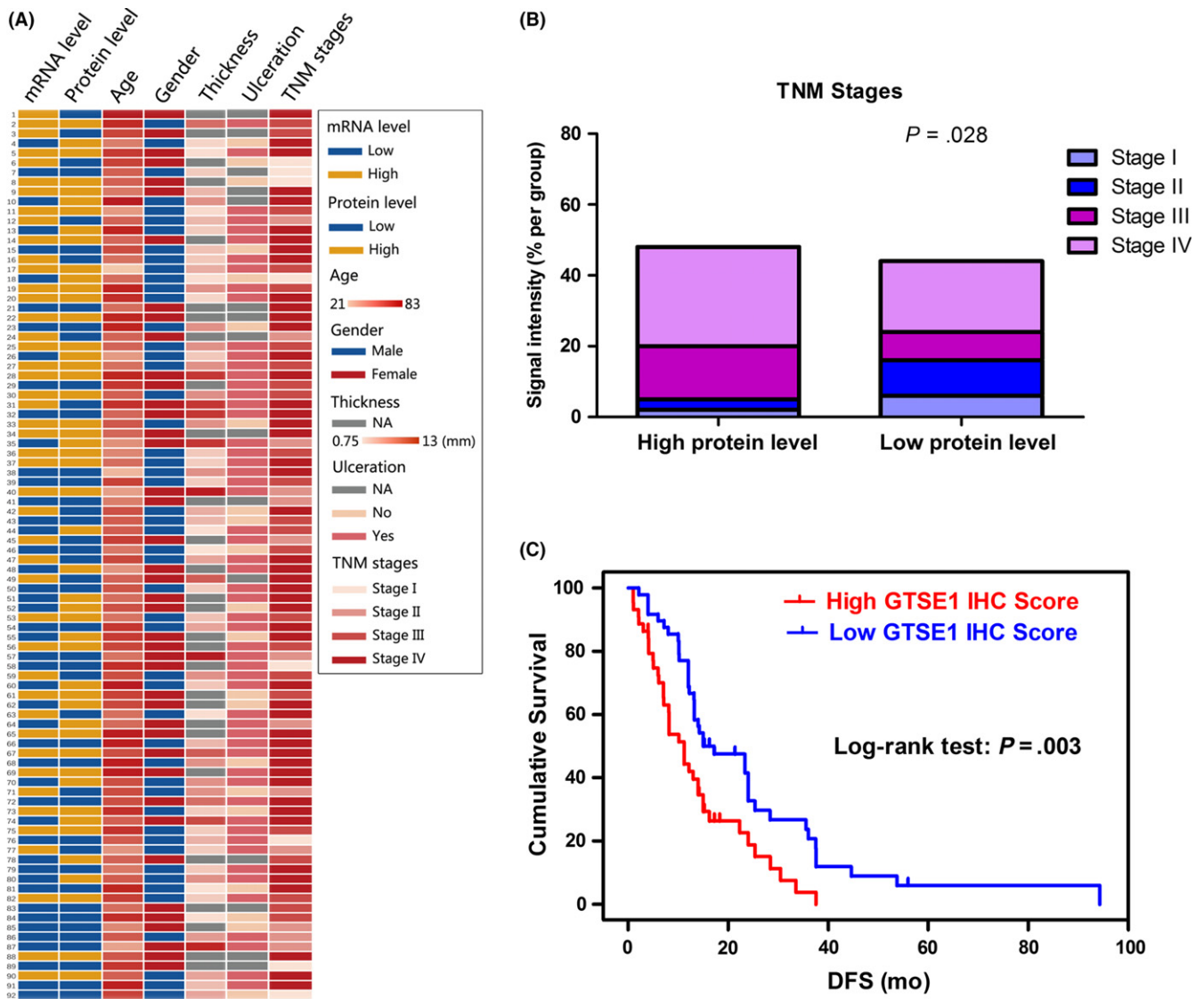
FIGURE 2 Clinical characteristics of 92 patients are summarized. A, G2 and S-phase expressed 1 (GTSE1) expression was correlated with acral melanoma (AM) progression. NA, not applicable; B, Patients with high GTSE1 expression tended to have higher clinical stages. C, Disease-free survival (DFS) of 92 patients with AM were compared between the low- and high-GTSE1 groups using the Kaplan-Meier method

3.3 | G2 and S-phase expressed 1 affects melanoma cell proliferation

To test our hypothesis that GTSE1 plays a role in AM progression, we transfected plasmids into AMC-1 and WM115 cells to overexpress GTSE1. GTSE1-depleted cells were also established by transient transfection of siRNA into AMC-2 and WM2664 cells. GTSE1 elevation and inhibition were confirmed at the protein level (Figure 3A,B). Cell proliferation was significantly enhanced in GTSE1-overexpressing cells compared to those transfected with the control vector ($P < .01$), and significantly decreased in GTSE1-depleted cells ($P < .01$; Figure 3C). We carried out flow cytometric analysis to evaluate whether GTSE1 affected cell cycle progression. As shown in Figure 3D, GTSE1 overexpression led a decreased proportion in the G0/G1 phase ($P < .05$) and an increased proportion in the G2/M

phase ($P < .01$), and GTSE1 depletion led to decreased G2/M cells compared to the controls ($P < .01$).

Next, we assessed whether GTSE1 affected tumor formation in vivo. We successfully constructed stable GTSE1-overexpressing clones in AMC-1 cells, and carried out tumorigenicity assays in 12 NOD-SCID mice with s.c. injection of 2×10^6 AMC-1-GTSE1 or control cells. Consistent with the in vitro results, GTSE1 overexpression significantly increased tumor growth rate compared to the control group ($P < .01$; Figure 3E-G).

3.4 | G2 and S-phase expressed 1 stimulates AM cell migration and invasion in vitro

To examine the role of GTSE1 in AM cell migration and invasion, wound healing and transwell assays were carried out. The wound-

TABLE 1 Cox regression analysis of GTSE1 protein expression and clinicopathological factors with DFS

Factor	Group	HR	95% CI	P-value
Univariate analysis				
Gender	Female vs Male	1.110	0.3631-1.641	.728
Age (y)	>60 vs ≤60	0.857	0.243-1.472	.622
TNM stage	III and IV vs I and II	2.320	1.437-3.746	<.001
Ulceration	Yes vs no	1.157	0.523-1.791	.365
Thickness	>2 mm vs ≤2 mm	1.713	1.072-2.738	.024
GTSE1 expression	High vs low	2.089	1.279-3.411	.003
Multivariate analysis				
TNM stage	III and IV vs I and II	2.885	1.593-4.228	<.001
GTSE1 expression	High vs low	2.000	1.252-3.195	.004

CI, confidence interval; DFS, disease-free survival; GTSE1, G2 and S-phase expressed 1; HR, hazard ratio.
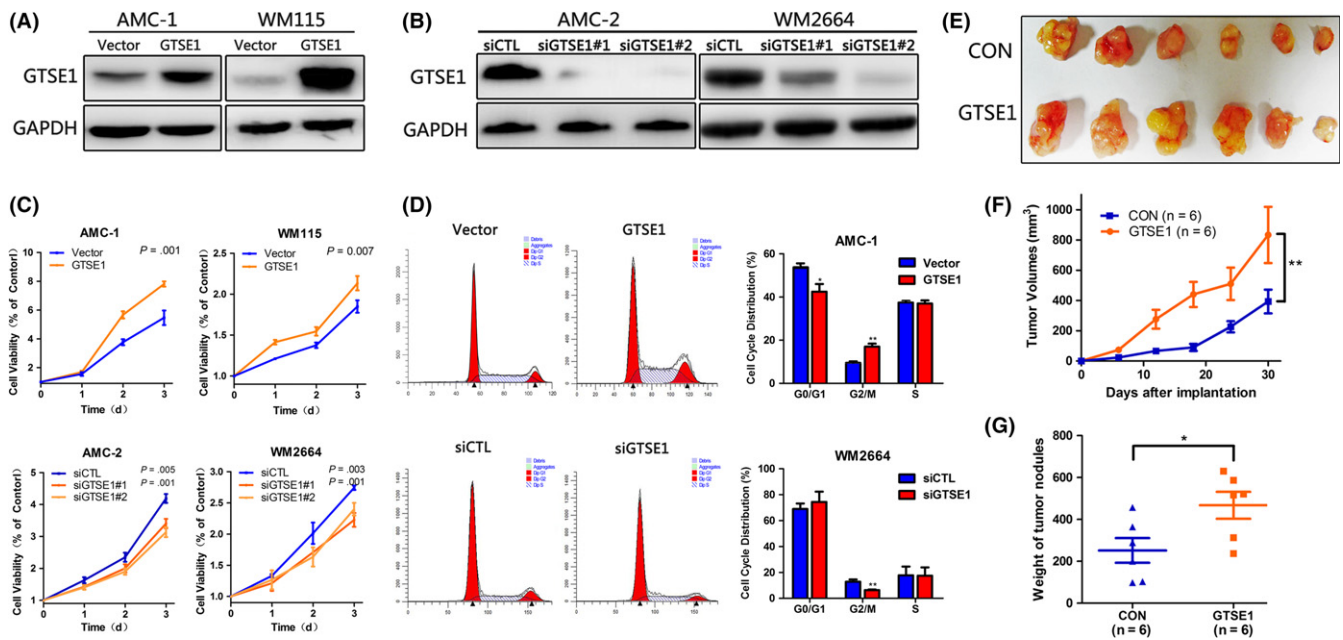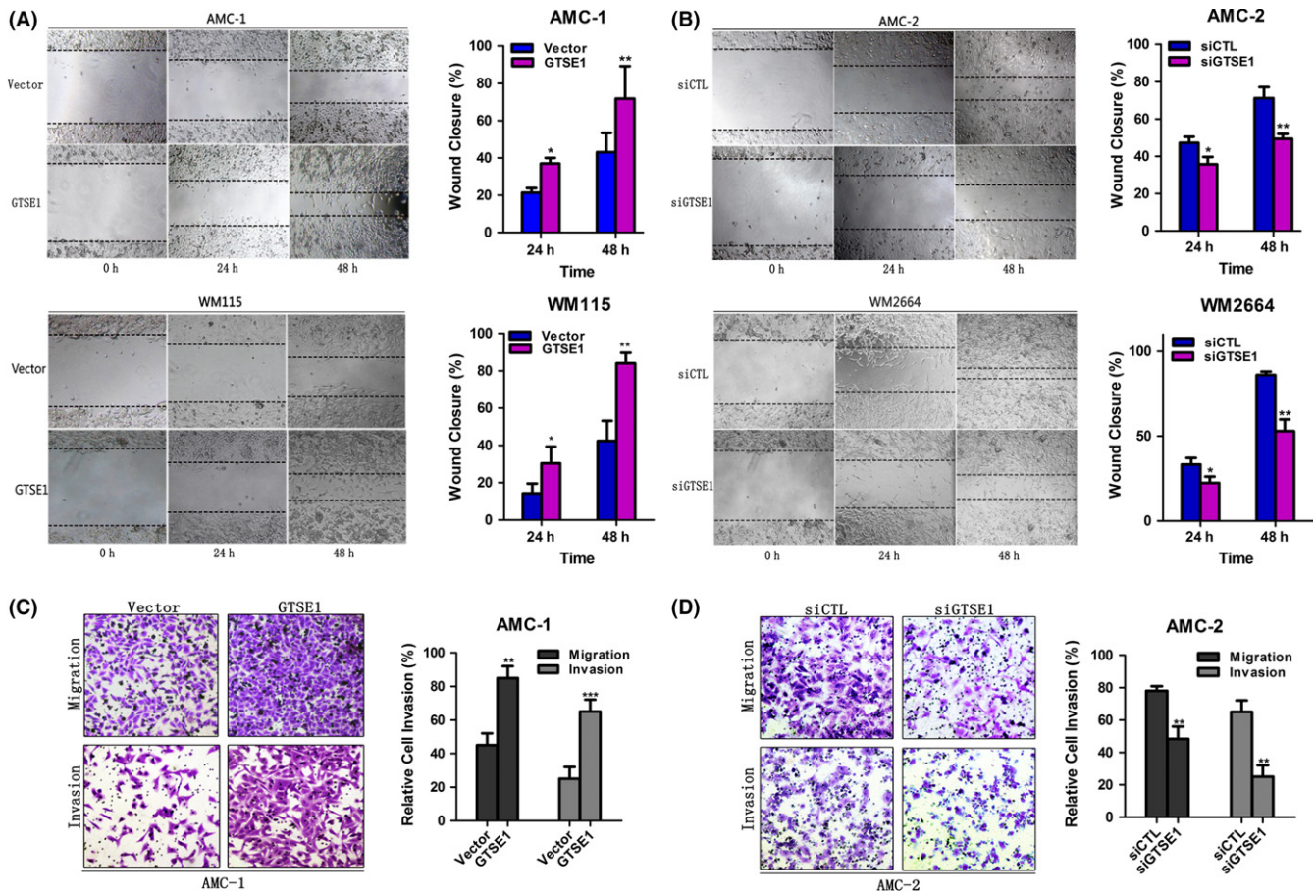

FIGURE 3 G2 and S-phase expressed 1 (GTSE1) affects acral melanoma (AM) cell proliferation. A,B, Western blots were carried out to confirm G2 and S-phase expressed 1 (GTSE1) overexpression in AMC-1 and WM115 cells and depletion in AMC-2 and WM2664 cells. C, Cell viability was examined with the CellTiter-Glo Luminescent Cell Viability assay, and the statistical significance of the growth curves was evaluated by repeated measures ANOVA. D, Cell cycle distribution was evaluated by flow cytometry. E, Representative pictures of subcutaneous implantation tumors formed from AMC-1-GTSE1 and AMC-1-control cells 30 d after injection. F,G, Comparison of tumor growth curves and tumor nodule weights was carried out at the indicated time points. Error bars indicate SD. *P < .05, ** P < .01

healing assay showed that ectopic expression of GTSE1 in AMC-1 and WM115 cells significantly promoted cell migration compared with vector-treated cells (P < .01; Figure 4A), and GTSE1 depletion in AMC-2 and WM2664 cells suppressed wound closure compared with siCTL cells (P < .01; Figure 4B). Transwell assays confirmed that ectopic expression of GTSE1 in AMC-1 cells promoted cell migration and invasion (P < .01; Figure 4C), whereas GTSE1 depletion in the highly metastatic AMC-2 line significantly decreased cell migration and invasion (P < .01; Figure 4D).

Altogether, the results suggest that GTSE1 stimulates AM cell migration and invasion.

3.5 | G2 and S-phase expressed 1 potentially facilitates metastasis in vivo

To investigate whether GTSE1 plays a role in AM metastasis, BLI using IVIS technology was used to monitor primary tumor growth and show the appearance of metastases. Luciferase-positive AMC-2-

FIGURE 4 G2 and S-phase expressed 1 (GTSE1) increases cell migration and invasion in acral melanoma (AM) cells. A,B Representative images from wound-healing assays. Original magnification, 100×. Histograms represent the wound closure rates at the indicated times. C,D, Migratory and invasive properties of AM cells were analyzed using a transwell filter with or without Matrigel coating. Original magnification, 100×. Error bars represent the mean ± SD of 3 independent experiments

shCTL or AMC-2-shGTSE1 cells were injected into NOD-SCID mice by the tail vein. Total bioluminescence was reduced in AMC-2-shGTSE1-injected mice compared to control mice 30 days after injection ($P < .001$; Figure 5A), and the lungs were resected and analyzed by H&E staining (Figure 5B). Therefore, GTSE1 can be considered a facilitator of metastasis.

3.6 | G2 and S-phase expressed 1 promotes EMT

We next examined whether GTSE1 functioned in promoting tumor progression by disrupting EMT, using both mesenchymal and epithelial markers (N-cadherin and E-cadherin, respectively). Concurrent with reduced migration and invasion abilities, GTSE1 depletion in AMC-2 and WM2664 resulted in increased E-cadherin and decreased N-cadherin, suggesting that GTSE1 is a driver of EMT in AM.

To explore molecular alterations causing these changes, we also examined expression of EMT-related transcription factors Snail, Slug, and Zeb1, which repress E-cadherin expression by directly binding the E-boxes of the E-cadherin promoter. Slug was downregulated in cells depleted of GTSE1 compared to control cells, whereas the expression of Snail and Zeb1 was unaffected (Figure 6).

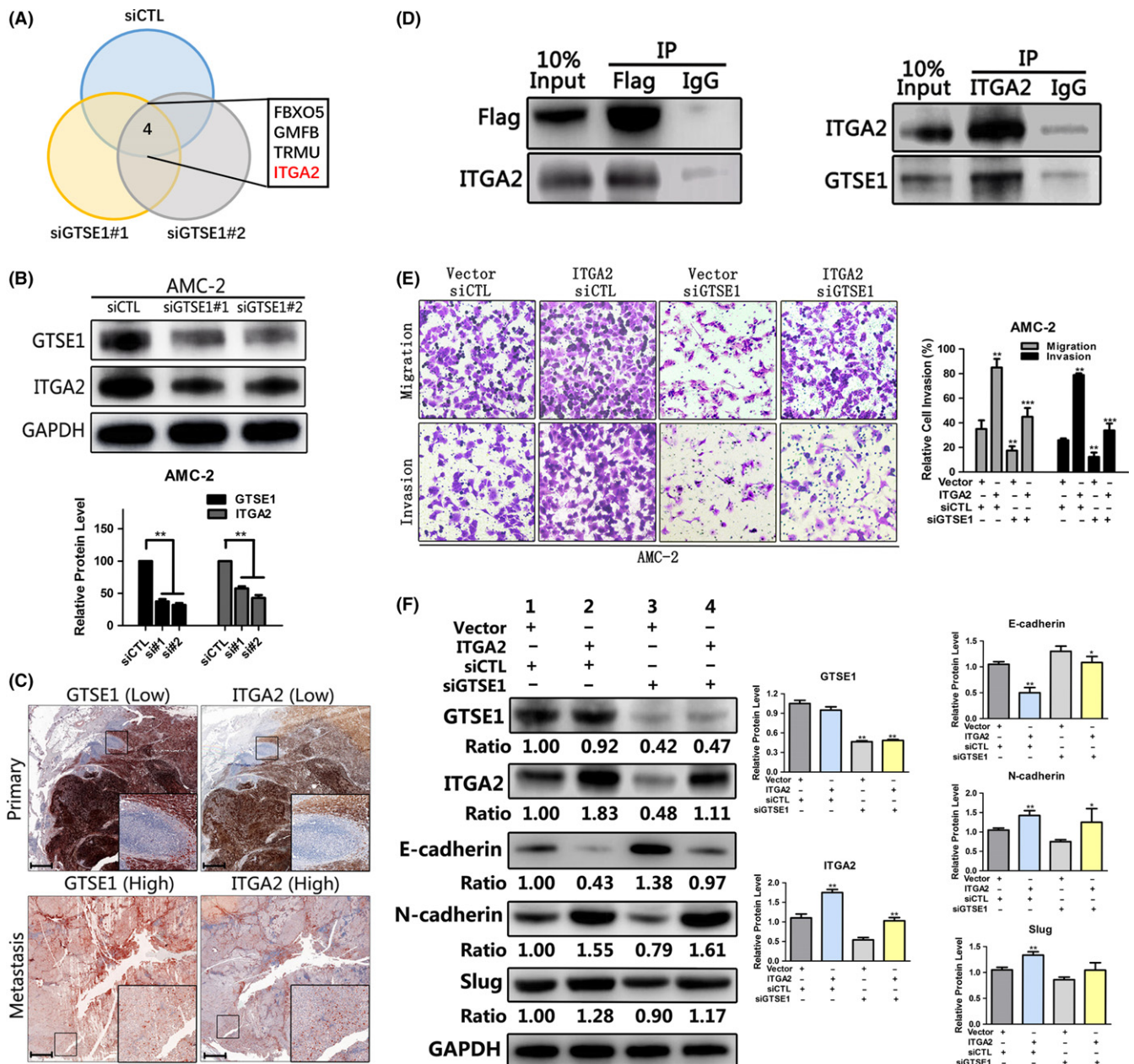## 3.7 | Integrin subunit alpha 2 is a downstream effector of GTSE1

As GTSE1 depletion led to increased E-cadherin and reduced N-cadherin and Slug, we further identified GTSE1-mediated changes on downstream signal transduction in the context of these EMT molecules. Transcriptome profiling analyses were carried out to explore variations in GTSE1 downstream effectors (Figure S1). Top 4 genes (F-box protein 5 [FBXO5], glia maturation factor beta [GMFB], tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase [TRMU], and ITGA2) the expression of which correlated positively with that of GTSE1, were verified by PCR in AMC-2 and WM2664 cell lines (Figures 7A, S2). Reduced ITGA2 expression was further confirmed after GTSE1 depletion in AMC-2 cells (Figure 7B). Figure 7C shows the positive correlation between ITGA2 and GTSE1 expression in situ. The correlation of these 2 protein expressions was further confirmed in 45 AM samples (Table 2, $P = .036$).

The observation led us to explore whether ITGA2 interacts with GTSE1. IP assays were carried out in AMC-2 cells, showing that FLAG-tagged GTSE1 immunoprecipitated ITGA2, and ITGA2 immunoprecipitated GTSE1, indicating a protein-protein interaction

FIGURE 5 G2 and S-phase expressed 1 (GTSE1) depletion inhibits acral melanoma (AM) cell metastasis in vivo. A, Left panel shows bioluminescence intensity analysis of mice 30 d after injection with AMC-2-shGTSE1 or AMC-2-shCTL cells. Right panel shows bioluminescent metastasis assays at the same time intervals. Data represent the mean ± SD. B, Representative H&E staining of lung tissues from the different groups. Magnification, ×400. ***$P < .001$

FIGURE 6 G2 and S-phase expressed 1 (GTSE1) regulates epithelial-mesenchymal transition (EMT) in acral melanoma (AM) cells. E-cadherin, N-cadherin, and Slug expression in GTSE1-depleted AMC-2 and WM2664 cell lines. GAPDH served as a loading control. Numbers below the panels represent normalized protein expression levels. Graphs on the right show quantification of western blot data from 3 independent experiments. **$P < .01$

(Figure 7D). To verify the involvement of ITGA2 in the GTSE1-mediated migration of AM cells, we cotransfected ITGA2-expressing or vector control plasmids into AMC-2 cells along with siGTSE1 or siCTL. As shown in Figure 7E, upregulation of ITGA2 expression rescued siGTSE1-mediated inhibition of AM migration. Western blot assays showed that ITGA2 also decreased E-cadherin and increased

FIGURE 7 Integrin subunit alpha 2 (ITGA2) is a downstream effector of G2 and S-phase expressed 1 (GTSE1). A,B, GTSE1 was found to regulate ITGA2 by transcriptome profiling and western blot analysis. FBXO5, F-box protein 5; GMFB, glia maturation factor beta; TRMU, tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase. C, Immunohistochemistry examination of acral melanoma (AM) tissues. Scale bars, 800 μm. Magnification, 5×. D, Immunoprecipitation (IP) of GTSE1 and ITGA2 followed by western blot analysis. E, In vitro transwell and invasion assays were conducted in the form of rescue experiments. Original magnification, 100×. Normalized ratios of migrated and invasive AMC-2 cells are shown on the right. Error bars represent the mean ± SD from 3 independent experiments. F, Expression of the indicated epithelial and mesenchymal markers were assessed in AMC-2 cells during the rescue assay. GAPDH served as a loading control. Numbers below the panels represent the normalized protein expression levels. Graphs on the right show the quantification of western blot data from 3 independent experiments. *$P < .05$, **$P < .01$, ***$P < .001$

N-cadherin and Slug expression in AMC-2 cells depleted of GTSE1 (Figure 7F).

4 | DISCUSSION

The strength of the present study is that it supplements the scarcity of acral melanoma research and provides novel insights into GTSE1

accelerating AM progression and its high expression correlates with poor clinical prognosis. As most AM patients are ineligible for current target therapy with vermurafenib or vermurafenib + dabrafenib[11] and imatinib,[14] GTSE1 provides hope for AM patients.

In the present study, we used AM cell lines and human tissue samples to confirm that GTSE1 expression was upregulated in metastatic AM. Importantly, we applied the clinical observation that

TABLE 2 Correlation of GTSE1 and ITGA2 expression

ITGA2 expression levels	GTSE1 expression levels	
	High (n = 26)	Low (n = 19)
High (n = 27)	19	8
Low (n = 18)	7	11
Consistency rate (%)	19/26 (73.1)	11/19 (57.8)
P-value[a]	.036	

GTSE1, G2 and S-phase expressed 1; ITGA2, integrin subunit alpha 2.
[a]Significance evaluated by chi-squared tests.

GTSE1 impacts AM progression. The cohort study of 92 patients indicated that higher GTSE1 expression strongly correlated with poorer clinical outcome with a shorter DFS. The cellular and molecular mechanisms by which GTSE1 promoted AM proliferation, migration, invasion, and metastasis were examined in AM cell lines. We found that GTSE1 functions as a stimulator of tumor migration and invasion by disrupting EMT. Our study provides novel insights into the role of GTSE1 in regulating EMT and migration/invasion in an ITGA2-dependent way, which is a downstream transcription factor.

Somatic point mutations and DNA amplification are regarded as the two main driving factors of the overexpression and activation of oncogenes.[33] Data from cBioPortal (available at http://www.cbioportal.org)[34,35] suggested that the frequency of *GTSE1* gene alterations in cutaneous melanoma is 4.61%-5.92%; gene mutations account for about 2.77% and gene amplification accounts for about 2.79% of these observed alterations. However, genetic aberrations of *GTSE1* have not been investigated in an AM cohort to date, and the mechanism underlying GTSE1 overexpression remains unclear. Further studies are required to verify the relationship between alterations of *GTSE1* and its high expression in metastatic AM.

The effects of GTSE1 on AM proliferation, migration, invasion, and metastasis highlight its importance in AM progression. GTSE1 regulates the cell cycle by interacting with p53 and repressing its ability to induce apoptosis.[20,28,29] Previous studies have investigated the expression and functional mechanisms of GTSE1 with respect to the tumor cell cycle.[36,37] Consistently, our investigation showed that upregulation of GTSE1 leads to increased G2/M phase in AMC-1 cells, and GTSE1 depletion decreased G2/M phase in WM2664 cells. These data emphasize that GTSE1 abrogates AM cell cycle checkpoints to accelerate proliferation. However, we did not examine whether this requires deactivation of p53.

Migration and invasion are important features controlling the metastasis of cancer cells, and these processes require changes to the microtubule cytoskeleton. In breast cancer, alteration of GTSE1 expression is associated with increased invasive potential, and GTSE1 was identified as a microtubule-associated plus-end tracking protein, which promotes cell migration through interactions with EB1.[31] Consistently, we have shown that migration and invasion of AM cells were stimulated by GTSE1 overexpression and suppressed by its depletion. These data suggest that upregulation of GTSE1 may be associated with increased metastatic

potential. The use of IVIS technology on NOD-SCID mice and lung micrometastases confirmed that GTSE1 depletion attenuated AM metastasis.

This observation prompted us to identify the underlying mechanisms of GTSE1 in migration, invasion, and metastasis. Increased cell migration and invasion are significant characteristics of EMT, which promotes the loss of tumor cell polarity and contact with neighboring cells, allowing cells to detach from the primary tumor and invade the local environment.[38,39] EMT has received considerable attention as a conceptual paradigm explaining invasion and cancer metastasis. Therefore, we explored EMT during AM tumor progression. EMT is defined by the loss of epithelial characteristics, such as a decrease in the expression of cell adhesion molecule E-cadherin and increased expression of N-cadherin.[40] As expected, depletion of GTSE1 in highly metastatic AM cell lines attenuated EMT. Our data are consistent with findings regarding the cadherin switch during malignant melanoma development.[41] Slug, a zinc finger transcription factor, has been proposed as a key EMT inducer affecting melanoma metastatic propensity.[42,43] A study of hepatocellular carcinoma also showed that GTSE1 depletion had a significant impact on EMT, causing decreased expression of Snail, N-Cadherin, and β-catenin and decreased metastatic potential.[24] In our data, decreased Slug occurred concomitantly with the cadherin switch and migration attenuation.

Our study also explored the downstream targets of GTSE1, and ITGA2 was found to be an interactor and downstream effector of GTSE1. Integrins are involved in the regulation of cell motility, migration, and invasion.[44] ITGA2, mainly together with the β1 integrin subunit, has also been reported in many cancers.[45-49] ITGA2 regulation by miRNAs and epigenetic modifications is crucial for invasion, metastasis and EMT.[50-53] Therefore, we explored the contribution of ITGA2 in siGTSE1-mediated cell AM migration and invasion. The results showed that ectopic ITGA2 expression can rescue siGTSE-1 mediated inhibition of AM progression, which is consistent with previous studies. Published data showed that ITGA2 is upregulated in osteosarcoma tissues, and ectopic expression of ITGA2 can decrease E-cadherin expression and increase the expression of N-cadherin, Vimentin, and Slug.[53] Likewise, our study showed that ectopic ITGA2 rescued siGTSE1-mediated inhibition of migration by disrupting EMT molecules. Taken together, 2 questions arise: (i) Is GTSE1 regulation of AM tumor progression ITGA2-dependent? (ii) Is ITGA2 also strongly correlated with AM clinical outcome? Future investigation is warranted to address these questions.

Although we provided novel insight to GTSE1 in AM progression, the present study has its limitations: (i) IVIS technology for metastasis and research on EMT phenotype was applied to GTSE1 inhibition cells only, and the present study was not able to provide validation of GTSE1 in gain-of-function cells. (ii) Although high GTSE1 expression correlates with poor prognosis, the study was not a preplanned prospective study and sample size was a bit small.

In summary, we have shown that GTSE1 promotes AM proliferation, migration, invasion and metastasis and correlates with clinical outcome. GTSE1 might represent a molecular target for AM therapy. Further investigation in disease models and clinical trials are needed.

CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

ORCID

Yan Kong (iD) http://orcid.org/0000-0002-6368-2112

REFERENCES

1. Piliang MP. Acral lentiginous melanoma. *Surg Pathol Clin*. 2009;2: 535-541.
2. Bradford PT, Goldstein AM, McMaster ML, Tucker MA. Acral lentiginous melanoma: incidence and survival patterns in the United States, 1986-2005. *Arch Dermatol*. 2009;145:427-434.
3. Wu XC, Eide MJ, King J, et al. Racial and ethnic variations in incidence and survival of cutaneous melanoma in the United States, 1999-2006. *J Am Acad Dermatol*. 2011;65:S26-S37.
4. Chi Z, Li S, Sheng X, et al. Clinical presentation, histology, and prognoses of malignant melanoma in ethnic Chinese: a study of 522 consecutive cases. *BMC Cancer*. 2011;11:85.
5. Furney SJ, Turajlic S, Stamp G, et al. The mutational burden of acral melanoma revealed by whole-genome sequencing and comparative analysis. *Pigment Cell Melanoma Res*. 2014;27:835-838.
6. Hayward NK, Wilmott JS, Waddell N, et al. Whole-genome landscapes of major melanoma subtypes. *Nature*. 2017;545:175-180.
7. Flaherty KT, Puzanov I, Kim KB, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med*. 2010;363:809-819.
8. Robert C, Karaszewska B, Schachter J, et al. Improved overall survival in melanoma with combined dabrafenib and trametinib. *N Engl J Med*. 2015;372:30-39.
9. Larkin J, Ascierto PA, Dréno B, et al. Combined vemurafenib and cobimetinib in BRAF-mutated melanoma. *N Engl J Med*. 2014;371: 1867-1876.
10. Carvajal RD, Antonescu CR, Wolchok JD, et al. KIT as a therapeutic target in metastatic melanoma. *JAMA*. 2011;305:2327-2334.
11. Si L, Kong Y, Xu X, et al. Prevalence of BRAF V600E mutation in Chinese melanoma patients: large scale analysis of BRAF and NRAS mutations in a 432-case cohort. *Eur J Cancer*. 2012;48:94-100.
12. Curtin JA, Fridlyand J, Kageshita T, et al. Distinct sets of genetic alterations in melanoma. *N Engl J Med*. 2005;353:2135-2147.
13. Si L, Guo J. C-kit-mutated melanomas: the Chinese experience. *Curr Opin Oncol*. 2013;25:160-165.
14. Kong Y, Si L, Zhu Y, et al. Large-scale analysis of KIT aberrations in Chinese patients with melanoma. *Clin Cancer Res*. 2011;17:1684-1691.
15. Braeuer RR, Watson IR, Wu CJ, et al. Why is melanoma so metastatic. *Pigment Cell Melanoma Res*. 2014;27:19-36.
16. Arozarena I, Wellbrock C. Targeting invasive properties of melanoma cells. *FEBS J*. 2017;284:2148-2162.
17. Hugo W, Zaretsky JM, Sun L, et al. Genomic and transcriptomic features of response to Anti-PD-1 therapy in metastatic melanoma. *Cell*. 2017;168:542.
18. Konieczkowski DJ, Johannessen CM, Abudayyeh O, et al. A melanoma cell state distinction influences sensitivity to MAPK pathway inhibitors. *Cancer Discov*. 2014;4:816-827.
19. Utrera R, Collavin L, Lazarević D, Delia D, Schneider C. A novel p53-inducible gene coding for a microtubule-localized protein with G2-phase-specific expression. *EMBO J*. 1998;17:5015-5025.
20. Monte M, Collavin L, Lazarevic D, Utrera R, Dragani TA, Schneider C. Cloning, chromosome mapping and functional characterization of a human homologue of murine gtse-1 (B99) gene. *Gene*. 2000;254: 229-236.
21. Zhang C, Min L, Zhang L, Ma Y, Yang Y, Shou C. Combined analysis identifies six genes correlated with augmented malignancy from non-small cell to small cell lung cancer. *Tumour Biol*. 2016;37:2193-2207.
22. Canevari RA, Marchi FA, Domingues MA, et al. Identification of novel biomarkers associated with poor patient outcomes in invasive breast carcinoma. *Tumour Biol*. 2016;37:13855-13870.
23. Pérez-Peña J, Alcaraz-Sanabria A, Nieto-Jiménez C, et al. Mitotic read-out genes confer poor outcome in luminal A breast cancer tumors. *Oncotarget*. 2017;8:21733-21740.
24. Wu X, Wang H, Lian Y, et al. GTSE1 promotes cell migration and invasion by regulating EMT in hepatocellular carcinoma and is associated with poor prognosis. *Sci Rep*. 2017;7:5129.
25. Penna I, Gigoni A, Costa D, et al. The inhibition of 45A ncRNA expression reduces tumor formation, affecting tumor nodules compactness and metastatic potential in neuroblastoma cells. *Oncotarget*. 2017;8:8189-8205.
26. Lee J, Sung CO, Lee EJ, et al. Metastasis of neuroendocrine tumors are characterized by increased cell proliferation and reduced expression of the ATM gene. *PLoS ONE*. 2012;7:e34456.
27. Zhou X, Temam S, Oh M, et al. Global expression-based classification of lymph node metastasis and extracapsular spread of oral tongue squamous cell carcinoma. *Neoplasia*. 2006;8:925-932.
28. Collavin L, Monte M, Verardo R, Pfleger C, Schneider C. Cell-cycle regulation of the p53-inducible gene B99. *FEBS Lett*. 2000;481:57-62.
29. Monte M, Benetti R, Buscemi G, Sandy P, Del SG, Schneider C. The cell cycle-regulated protein human GTSE-1 controls DNA damage-induced apoptosis by affecting p53 function. *J Biol Chem*. 2003;278:30356-30364.
30. Monte M, Benetti R, Collavin L, Marchionni L, Del SG, Schneider C. hGTSE-1 expression stimulates cytoplasmic localization of p53. *J Biol Chem*. 2004;279:11744-11752.
31. Scolz M, Widlund PO, Piazza S, et al. GTSE1 is a microtubule plus-end tracking protein that regulates EB1-dependent cell migration. *PLoS ONE*. 2012;7:e51259.
32. Kong Y, Sheng X, Wu X, et al. Frequent genetic aberrations in the CDK4 pathway in acral melanoma indicate the potential for CDK4/6 inhibitors in targeted therapy. *Clin Cancer Res*. 2017;23:6946-6957.
33. Zhang F, Gu W, Hurles ME, Lupski JR. Copy number variation in human health, disease, and evolution. *Annu Rev Genomics Hum Genet*. 2009;10:451-481.
34. Cerami E, Gao J, Dogrusoz U, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov*. 2012;2:401-404.
35. Gao J, Aksoy BA, Dogrusoz U, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal*. 2013;6:pl1.
36. Guo L, Zhang S, Zhang B, et al. Silencing GTSE-1 expression inhibits proliferation and invasion of hepatocellular carcinoma cells. *Cell Biol Toxicol*. 2016;32:263-274.

37. Bublik DR, Scolz M, Triolo G, Monte M, Schneider C. Human GTSE-1 regulates p21(CIP1/WAF1) stability conferring resistance to paclitaxel treatment. *J Biol Chem*. 2010;285:5274-5281.

38. Alonso SR, Tracey L, Ortiz P, et al. A high-throughput study in melanoma identifies epithelial-mesenchymal transition as a major determinant of metastasis. *Cancer Res*. 2007;67:3450-3460.

39. Johnson JP. Cell adhesion molecules in the development and progression of malignant melanoma. *Cancer Metastasis Rev*. 1999;18:345-357.

40. Hay ED. Role of cell-matrix contacts in cell migration and epithelial-mesenchymal transformation. *Cell Differ Dev*. 1990;32:367-375.

41. Kuphal S, Bosserhoff AK. Influence of the cytoplasmic domain of E-cadherin on endogenous N-cadherin expression in malignant melanoma. *Oncogene*. 2006;25:248-259.

42. Fenouille N, Tichet M, Dufies M, et al. The epithelial-mesenchymal transition (EMT) regulatory factor SLUG (SNAI2) is a downstream target of SPARC and AKT in promoting melanoma cell invasion. *PLoS ONE*. 2012;7:e40378.

43. Poser I, Domínguez D, de Herreros AG, Varnai A, Buettner R, Bosserhoff AK. Loss of E-cadherin expression in melanoma cells involves up-regulation of the transcriptional repressor Snail. *J Biol Chem*. 2001;276:24661-24666.

44. Aplin AE, Howe AK, Juliano RL. Cell adhesion molecules, signal transduction and cell growth. *Curr Opin Cell Biol*. 1999;11:737-744.

45. Augoff K, Das M, Bialkowska K, McCue B, Plow EF, Sossey-Alaoui K. miR-31 is a broad regulator of β1-integrin expression and function in cancer cells. *Mol Cancer Res*. 2011;9:1500-1508.

46. Rathinam R, Alahari SK. Important role of integrins in the cancer biology. *Cancer Metastasis Rev*. 2010;29:223-237.

47. Yoshimura K, Meckel KF, Laird LS, et al. Integrin alpha2 mediates selective metastasis to the liver. *Cancer Res*. 2009;69:7320-7328.

48. Zhang Z, Ramirez NE, Yankeelov TE, et al. alpha2beta1 integrin expression in the tumor microenvironment enhances tumor angiogenesis in a tumor cell-specific manner. *Blood*. 2008;111:1980-1988.

49. Dong J, Wang R, Ren G, et al. HMGA2-FOXL2 axis regulates metastases and epithelial-to-mesenchymal transition of chemoresistant gastric cancer. *Clin Cancer Res*. 2017;23:3461-3473.

50. Chin SP, Marthick JR, West AC, et al. Regulation of the ITGA2 gene by epigenetic mechanisms in prostate cancer. *Prostate*. 2015;75:723-734.

51. Zhao X, Wu Y, Lv Z. miR-128 modulates hepatocellular carcinoma by inhibition of ITGA2 and ITGA5 expression. *Am J Transl Res*. 2015;7:1564-1573.

52. Nones K, Waddell N, Song S, et al. Genome-wide DNA methylation patterns in pancreatic ductal adenocarcinoma reveal epigenetic deregulation of SLIT-ROBO, ITGA2 and MET signaling. *Int J Cancer*. 2014;135:1110-1118.

53. Liu X, Liang Z, Gao K, et al. MicroRNA-128 inhibits EMT of human osteosarcoma cells by directly targeting integrin α2. *Tumour Biol*. 2016;37:7951-7957.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.