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Epithelial-mesenchymal transition may be involved in the immune evasion of circulating gastric tumor cells via downregulation of ULBP1

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Abstract

Background: Increasing numbers of studies have demonstrated that circulating tumor cells (CTCs) undergo a phenotypic change termed epithelial-mesenchymal transition (EMT), and researchers have proposed that EMT might provide CTCs with increased potential to survive in the different microenvironments encountered during metastasis through various ways, such as by increasing cell survival and early colonization. However, the exact role of EMT in CTCs remains unclear.

Methods: In this study, we identified CTCs of 41 patients with gastric cancer using Cyttel-CTC and im-FISH (immune-fluorescence in situ hybridization) methods, and tested the expression of EMT markers and ULBP1 (a major member of the NKG2D—natural killer [NK] group 2 member D—ligand family) on CTCs. Moreover, we investigated the relationship between the expression of EMT markers and ULBP1 on CTCs and gastric cancer cell lines.

Results: Our results showed that the CTCs of gastric cancer patients exhibited three EMT marker subtypes, and that the expression of ULBP1 was significantly lower on mesenchymal phenotypic CTCs (M^+CTCs) than on epithelial phenotypic CTCs (E^+CTCs). EMT induced by TGF-β in vitro produced a similar phenomenon, and we therefore proposed that EMT might be involved in the immune evasion of CTCs from NK cells by altering the expression of ULBP1.

Conclusions: Our study indicated that EMT might play a vital role in the immune invasion of CTCs by regulating the expression of ULBP1 on CTCs. These findings could provide potential strategies for targeting the immune evasion capacity of CTCs.

Hu and Tian equally contributed to this study.

1 | INTRODUCTION

Gastric cancer is among the most common malignant digestive system tumors worldwide.[1] Despite advances in the prevention, detection, and adjuvant treatment of gastric cancer, the number of people diagnosed with gastric cancer is increasing, and metastasis remains a major causative factor of cancer-related death.[2-5] However, the mechanism underlying metastasis remains unclear. Recently, studies have demonstrated that metastasis is a multi-step process that involves the presence of circulating tumor cells (CTCs) in the bloodstream and their growth in other organs.[6-8] CTCs are singular cell or small cell masses that disseminate from primary tumors into blood vessels, and are usually identified by the following criteria: (a) atypical phenotype, with high nuclear to cytoplasm ratio or irregular shape; (b) intratumor heterogeneity, including the amplification of aneuploidy in chromosomes, altered cell-surface antigen markers, molecular diversity, and varying mobility potential.[9-11] Since the first reports on CTCs, the enumeration and analysis of the cells have attracted increasing attention due to their crucial roles in the metastatic cascade and the progression of metastasis. At present, CTC detection, via a novel method termed "liquid biopsies," is widely used in the clinical treatment of various malignant tumor types.[12-15] Compared to traditional tissue biopsy of solid neoplasms, noninvasive liquid biopsies are considered to be superior, and enable clinicians to use blood samples of only a few milliliters from cancer patients to perform "real-time" monitoring and analysis of the cancer, to obtain more information at an earlier stage.[16-18] Recently, studies have revealed that CTCs of gastric cancer move from the primary tumor into the bloodstream and travel to other organs during the early stage, thus exerting a dominant contribution to early metastasis.[19,20] In this process, CTCs undergo a phenotypic change termed epithelial-mesenchymal transition (EMT), in which the cells acquire a mesenchymal phenotype and thus become more aggressive and invasive.[21-23] Moreover, researchers have proposed that EMT might provide CTCs with increased potential to survive in the different microenvironments encountered during metastasis through various ways, such as by enhancing metastatic competence, by increasing cell survival and early colonization, by promoting the formation of specific protective cytoskeletal structures and clusters of CTCs, and by promoting immune evasion.[24-27] However, despite extensive experimental data, the exact role of EMT remains unclear, particularly regarding CTC immune evasion in the peripheral bloodstream.

The present study aimed to explore the role of EMT in the immune escape of gastric CTCs. We used Cyttel-CTC and im-FISH (immune-fluorescence in situ hybridization) methods to detect CTCs and to investigate the expression of EMT markers and ULBP1 on CTCs.[28-30] Moreover, we investigated the relationship between the expression of EMT markers and ULBP1 in vitro in gastric cancer cell lines. Our results suggested that the expression of ULBP1 was significant lower on mesenchymal phenotypic CTCs than on epithelial phenotypic CTCs, and that EMT induced by TGF-β in vitro could produce a similar change. We therefore proposed that EMT might be involved in the immune escape of CTCs from natural killer (NK) cell killing through altered expression of ULBP1 on the tumor cells. To the best of our knowledge, this is the first study to analyze the EMT phenomenon combined with ULBP1 expression status in circulating gastric carcinoma cells.

2 | MATERIAL AND METHODS

2.1 | Patients and sample collection

A total of 41 patients with newly diagnosed gastric cancer from the Department of Gastrointestinal Surgery of Binzhou Medical University Hospital (Binzhou City, China) were included in the study from July to September 2016. The diagnosis was confirmed by histopathological analysis and the patients did not receive any treatments, such as neoadjuvant chemical therapy or radiotherapy, prior to sample collection. 3.2-mL peripheral blood samples were collected using 5-mL tubes with ACD anti-coagulant (BD Technology; 0.8 mL) after discarding the first 2 mL to avoid potential skin cell contamination from the venipuncture. The blood was stored at room temperature for no more than 24 hours for further analysis. Clinical information on the patients, including age and gender, was collected. The Medical Ethical Committee of the Affiliated Hospital of Binzhou Medical University approved this research, and written informed consent was obtained from the patients prior to blood collection.

2.2 | Enrichment of CTCs in peripheral blood

A technique based on karyotypic in situ characterization of CTCs was used to detect CTCs in peripheral blood. The procedure was similar to that reported previously by Ge et al.[31] In

brief, 3.2-mL blood samples were collected in 5-mL tubes, then transferred into 50-mL tubes containing 40 mL CS1 working solution (Cyttel) after thorough mixing. Subsequently, the solution was centrifuged at 500× g for 5 minutes at room temperature. Supernatants were discarded and CS2 working buffer (Cyttel) was added into the tubes to remove the red blood cells. CS3 working buffer (Cyttel) and magnetic beads (Cyttel) were subsequently used to deplete the majority (>99.9%) of leukocytes by magnetic separation and gradient centrifugation. The middle cell layer containing rare cells was transferred to another tube for further centrifugation. Following centrifugation, the upper liquid (100 μL) was discarded and 200 μL CF1 stationary liquid (Cyttel) was added to resuspend the remaining cells. Finally, the cells were applied onto coated CTC PEN membrane slides and dried overnight at room temperature for immediate use or storage at −20°C.

2.3 | Combined immunocytochemistry staining and FISH

After the dry slides were dehydrated in an ethanol series (75%, 85%, and 100%; 3 minutes each), the slides were incubated with a cocktail of Alexa Fluor 594-conjugated anti-CD45 (Cytelligen) for 1 hour in the dark. Subsequently, FISH was performed with centromere probe 8 (orange fluorescent) (Abbott Molecular Diagnostics) and 17 (green fluorescent) (Abbott Molecular Diagnostics) using an s500 Statspin ThermoBrite Slide Hybridization/Denaturation System (Abbott Molecular) for 1.5 hours at 37°C. Finally, the slides were mounted with mounting media containing 4'-6-diamidino-2-phenylindole (DAPI) (Life Technology) and analyzed with an automatic fluorescent microscope using a 40× objective lens (Olympus BX53). Genomic aberrations and negative expression of CD45 are hallmarks of malignant cells, and CTCs were identified as CEP8$^+$/CD45$^-$/DAPI$^+$ or CEP17$^+$/CD45$^-$/DAPI$^+$.

2.4 | Detection of Vimentin, EpCAM, and ULBP1 surface expression on CTCs

The identified CTCs were isolated using a laser capture microdissection system-Leica LMD6500 (Leica). After fixation and dehydration, the CTCs were incubated with a cocktail of Vimentin RNA probe-labeled AlexaFluor594 (Invitrogen), EpCAM RNA probe-labeled AlexaFluor488 (Invitrogen) and ULBP1 RNA probe-labeled AlexaFluor647 (Invitrogen) (capture probe sequences are shown in table 4) in the s500 Statspin ThermoBrite Slide Hybridization/Denaturation System for 1.5 hours at 37°C. Subsequently, the CTCs were washed three times with phosphate-buffered saline (PBS), then mounted with DAPI (Life Technology) containing mounting media,

and subjected to image analysis using a three-color fluorescence microscope.

2.5 | Cell culture and EMT induction

The human gastric carcinoma cell line SGC7901 was obtained from the Center Laboratory of Binzhou Medical University. Cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific, Inc) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc) and 2% penicillin/streptomycin in a humidified atmosphere with 95% air and 5% CO_2, in an incubator (Thermo Fisher Scientific, Inc) at 37°C. EMT was induced via the application of exogenous recombinant human TGF-β1 (Thermo Fisher Scientific, Inc) at concentrations of 5, 10, and 20 ng/mL. The cells were collected for subsequent analysis at 72 hours after the addition of TGF-β1.

2.6 | Western blot analysis

After the total protein was extracted and quantitated using a BCA Protein Assay Kit (Solarbio), equal amounts of protein (30-50 μg per lane) were subjected to 10% sodium dodecyl sulfate-tris glycine polyacrylamide gel electrophoresis (Solarbio), then electroblotted on to a polyvinylidene fluoride membrane (Solarbio). After blocking in 5% nonfat milk (BD Technology), the membrane was incubated with primary antibodies against EpCAM, Vimentin, MICA, and ULBP1/2 (Abcam Technology) at dilutions of 1:1000-2000 overnight, then with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (ZSGB-BIO) at a dilution of 1:10 000-20 000. Immunocomplexes were visualized using an enhanced chemiluminescence detection system (Billerica, MA, USA). GAPDH (GOOD HERE Technology) was used as an internal reference for comparison with the other proteins.

2.7 | Immunofluorescence

Following treatment with 20 ng/mL TGF-β1 for 72 hours, cultured SGC7901 cells on glass chamber slides (Thermo Fisher Scientific, Inc) were fixed with 4% paraformaldehyde in PBS (Beyotime) for 15 minutes at room temperature, and then were washed three times with PBS. Subsequently, the fixed cells were permeabilized with 0.5% Triton X-100 (Solarbio) in PBS for 10 minutes, then blocked with 1% BSA in PBS for 1 hour. Following this, the cells were incubated with a cocktail of primary antibodies against EpCAM, Vimentin, MICA, and ULBP1 (Abcam Technology) overnight at 4°C. The cells were then washed with PBS and incubated with Dylight 488/549-conjugated secondary antibody for 1 hour at room

temperature. Finally, the slides were mounted with mounting media containing DAPI (Life Technology) and analyzed with an automatic fluorescent microscope using a 40× objective lens (Olympus BX53).

2.8 | Statistical analysis

Statistical analyses were performed using Statistical Package for Social Science Statistics 22.0 software (SPSS; IBM) and GraphPad Prism Software version 5.0 (San Diego, CA). The chi-square test or Fisher's exact test were used to assess associations between CTC positivity and clinicopathological characteristics of the patients. One-way ANOVA was used to evaluate the differences among groups. A value of $P < .05$ was considered to indicate a statistical significance.

3 | RESULTS

3.1 | Patient characteristics and detected gastric CTCs

As shown in Table 1, a total of 41 gastric cancer patients were included in this study: 30 males and 11 females, with a mean age of 64.37 ± 8.75 and 63.00 ± 7.27, respectively. CTCs were detected in 29 of the 41 patients (70.7%), and the CTC counts ranged from 0 to 13 cells/3.2 mL peripheral blood. As shown in Figure 1, CTCs were identified as polyploid CEP8$^+$/CD45$^-$/DAPI$^+$ or polyploid CEP17$^+$/CD45$^-$/DAPI$^+$ cells, while white blood cells (WBCs) were identified as diploid CEP8$^+$/CD45$^+$/DAPI$^+$ and diploid CEP17$^+$/CD45$^+$/DAPI$^+$ cells. We defined a CTC number of $\geq$2/3.2 mL peripheral blood as positive detection.

3.2 | EMT markers expressed on gastric CTCs

To evaluate EMT phenotype in CTCs, we assessed the expression of EMT markers, including EpCAM and Vimentin, on the identified CTCs. As shown in Figure 2, we classified CTCs into three subpopulations based on the expression of EMT markers: Epithelial CTCs (Figure 2A, E$^+$CTC), mesenchymal CTCs (Figure 2C, M$^+$CTC) and biphenotypic (Figure 2B, E$^+$/M$^+$CTC) CTCs. These three types of CTCs exhibited high-level expression of EpCAM, Vimentin, and both EpCAM and Vimentin, respectively. As shown in Table 2, E$^+$CTCs were the most commonly identified subtype, with a positive rate of 93.1%, and were detected in the majority of CTC-positive gastric patients. The second most common subtype was E$^+$/M$^+$CTC, with a positive rate of 86.21%. M$^+$CTCs were identified in 22

TABLE 1 Detected CTCs in 41 gastric cancer patients

Patients	Gender	Age	CTCs count (n)
1	F	48	3
2	M	68	5
3	M	68	4
4	M	44	4
5	M	65	13
6	M	66	2
7	M	62	2
8	M	74	0
9	M	57	2
10	M	51	3
11	M	78	0
12	M	80	2
13	M	61	0
14	M	50	3
15	F	73	0
16	M	70	12
17	F	72	1
18	M	64	8
19	F	64	6
20	M	72	1
21	M	61	6
22	M	62	9
23	F	62	6
24	M	65	12
25	M	50	2
26	M	58	1
27	M	82	3
28	M	67	0
29	F	64	2
30	F	62	8
31	F	64	0
32	M	68	2
33	M	60	6
34	M	71	4
35	F	53	0
36	M	66	0
37	F	64	1
38	M	69	7
39	M	62	4
40	F	67	4
41	M	60	4

Abbreviations: CTCs, circulating tumor cells; F, female; M, male; n, number.

of the 29 CTC-positive patients, with a positive rate of 75.86%. The count ranges of the three CTC subpopulations were 0-6, 0-5, and 0-4 cells/3.2 mL peripheral blood,
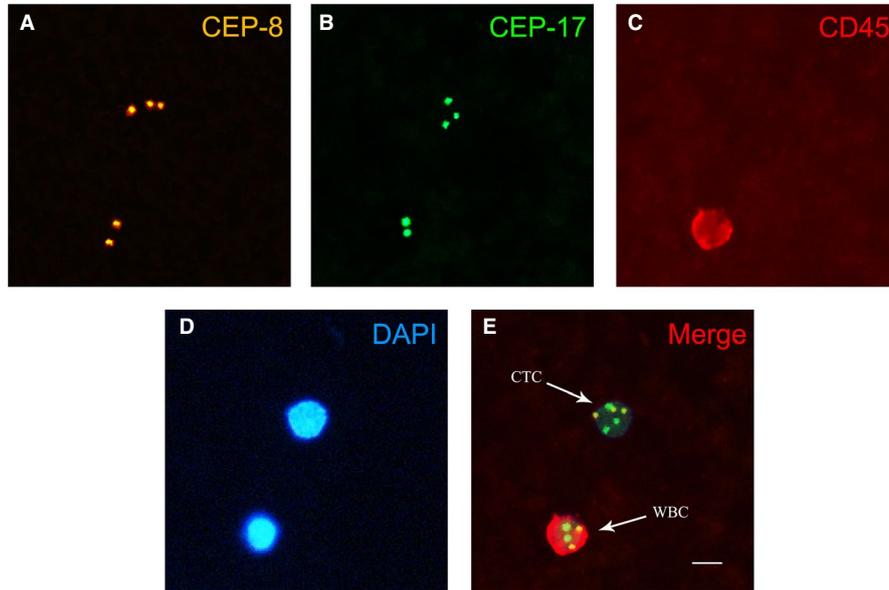
FIGURE 1 Detected circulating tumor cells (CTCs) in the peripheral blood of gastric cancer patients. A, CEP-8 probe signal staining (yellow) in CTCs (three points) and white blood cells (WBCs) (two points). B, CEP-17 probe signal staining (green) in CTCs (three points) and WBCs (two points). C, CD45 staining (red) in CTCs (negative) and WBCs (positive). D, DAPI staining (blue) in CTCs and WBCs. E, Merged image with probe signals and CD45 and nuclear staining; CTCs were identified as polyploid CEP8$^+$/CD45$^-$/DAPI$^+$ or polyploid CEP17$^+$/CD45$^-$/DAPI$^+$ cells, and WBCs were identified as diploid CEP8$^+$/CD45$^+$/DAPI$^+$ or diploid CEP17$^+$/CD45$^+$/DAPI$^+$ cells. Scale bar = 10 μm

respectively. Notably, the patients that presented with higher CTC counts seemed to exhibit higher counts of EMT-phenotypic CTCs.

3.3 | ULBP1 expression was downregulated on E$^+$/M$^+$ and M$^+$ CTCs

It is established that NKG2D ligands play a vital role in immune responses mediated by NK cells and CTLs, and that ULBP1 is a major member of the NKG2D ligand family. To further investigate the role of EMT in the immune evasion capacity of CTCs, we detected the expression of ULBP1 on the identified CTCs. For each of the different CTC subpopulations exhibiting the EMT phenotype, there were significant differences in the expression levels of ULBP1. Notably, the expression level of

ULBP1 was substantially lower in the M$^+$CTC subtype compared with the E$^+$/M$^+$ CTC and E$^+$ CTC subtypes (Figure 3).

3.4 | TGF-β1-induced EMT attenuates the expression of ULBP1 in gastric SGC7901 cells

TGF-β1 is considered to be a prototypical cytokine inducer of EMT. The above results showed that ULBP1 surface expression was downregulated on M$^+$CTCs. To identify the association between EMT and the expression of ULBP1, we investigated whether the expression of ULBP1 was modulated during the stimulation of EMT with exogenous TGF-β1 on gastric SGC7901 cells in vitro. As shown in Figure 4A, after treatment with exogenous TGF-β1 (5, 10, and 20 ng/mL) for 72 hours, a decrease in EpCAM expression, together with an
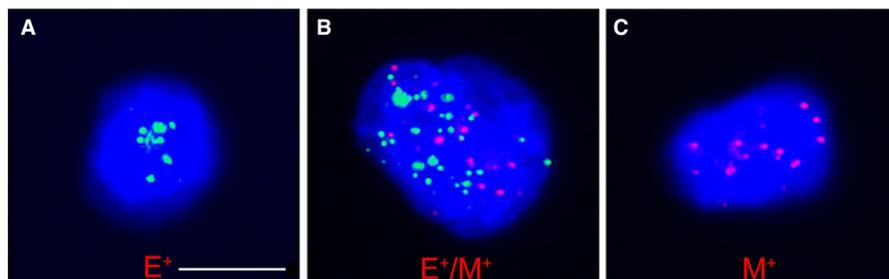

FIGURE 2 Three epithelial-mesenchymal transition subtypes of circulating tumor cells (CTCs) in gastric cancer patients. Epithelial biomarkers (EpCAM) are indicated by green dots; mesenchymal biomarkers (Vimentin) are indicated by red dots. Cells with green dots represent epithelial CTCs (A), cells with red dots represent mesenchymal CTCs (C), and cells with red and green dots represent biphenotypic CTCs (B). Scale bar = 10 μm

TABLE 2 Counts of EMT phenotypic CTCs in CTCs positive patients

Patients	Gender	Age	CTCs count (n)	E$^+$CTCs count (n)	E$^+$/M$^+$CTCs count (n)	M$^+$CTCs count (n)
1	M	66	2	1	1	0
2	M	62	2	2	0	0
3	M	57	2	1	1	0
4	M	80	2	2	0	0
5	M	50	2	1	1	0
6	F	64	2	1	0	1
7	M	68	2	0	0	2
8	F	48	3	1	2	0
9	M	51	3	2	1	0
10	M	50	3	1	1	1
11	M	82	3	0	2	1
12	M	68	4	1	2	1
13	M	44	4	1	1	2
14	M	71	4	2	1	1
15	M	62	4	1	2	1
16	F	67	4	2	1	1
17	M	60	4	1	1	2
18	M	68	5	2	1	2
19	F	64	6	3	1	2
20	M	61	6	2	3	1
21	F	62	6	2	2	2
22	M	60	6	1	3	2
23	M	69	7	2	2	3
24	M	64	8	4	2	2
25	F	62	8	3	3	2
26	M	62	9	4	2	3
27	M	70	12	3	5	4
28	M	65	12	4	5	3
29	M	65	13	6	4	3

Abbreviations: CTCs, circulating tumor cells; E$^+$/M$^+$CTCs, biophenotypic CTCs; E$^+$CTCs, epithelial CTCs; EMT, epithelial-mesenchymal transition; F, female; M, male; M$^+$CTCs, mesenchymal CTCs.

increase in Vimentin protein levels, was observed on SGC7901 cells in a dose-dependent manner (Figure 4A). We then performed immunofluorescence at 72 hours posttreatment with 20 ng/mL TGF-β1, and EMT phenotype was observed in the SGC7901 cells (Figure 4B). Similarly, ULBP1 expression was also measured by western blotting and immunofluorescence, and significantly decreased levels of ULBP1 were detected on SGC7901 cells (Figure 5A,B). These results indicated that EMT might be involved in regulating the expression of ULBP1.

4 | DISCUSSION

In previous decades, efforts have been made to develop technologies for distinguishing CTCs from the billions of nonmalignant blood cells present in the blood.[32-35] At present, the isolation and enumeration of CTCs from peripheral blood is considered to be a potential strategy for assessing disease progression and response to therapies, due to its numerous advantages.[36-39] Currently, methods of CTC isolation are mostly performed according to cell biological properties, physical properties, or a combination of both.[40,41] Other methods have also been developed by incorporating multiple principles to achieve optimal cell isolation.[42] In the current study, we used a negative enrichment technique based on magnetic separation and gradient centrifugation to capture CTCs in peripheral blood samples from gastric cancer patients.[43,44] Unlike other negative enrichment methods, these magnetic separation and gradient centrifugation methods could deplete WBCs effectively,
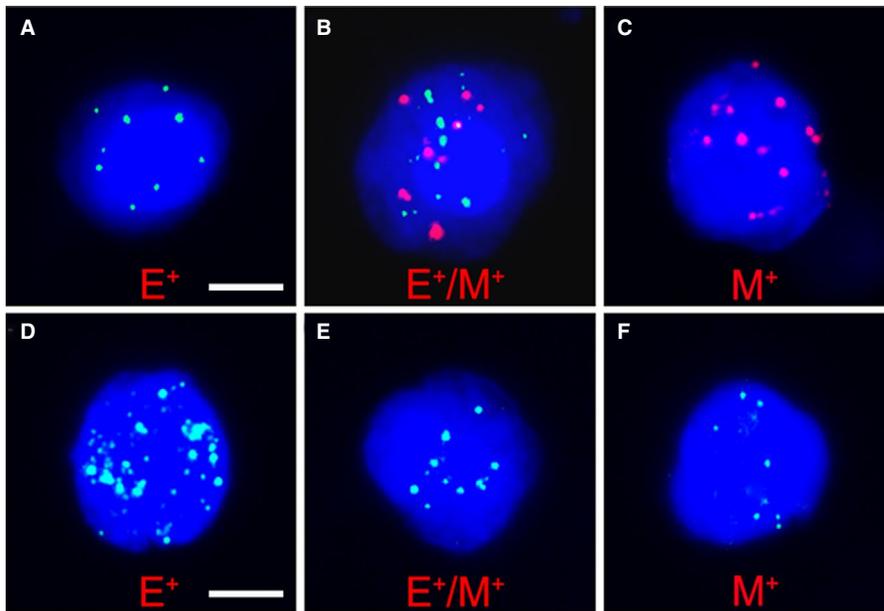
FIGURE 3 Surface expression of ULBP1 on circulating tumor cells (CTCs) with different epithelial-mesenchymal transition (EMT) phenotypes. A-C, Expression of EpCAM and Vimentin on predetermined CTCs (blue dots: epithelial biomarker expression; red dots: mesenchymal biomarker expression; scale bars = 5μm). D-F, ULBP1 expression on different EMT-phenotypic CTCs (bright blue dots: ULBP1 expression; scale bar = 10 μm). *P < .05, **P < .01
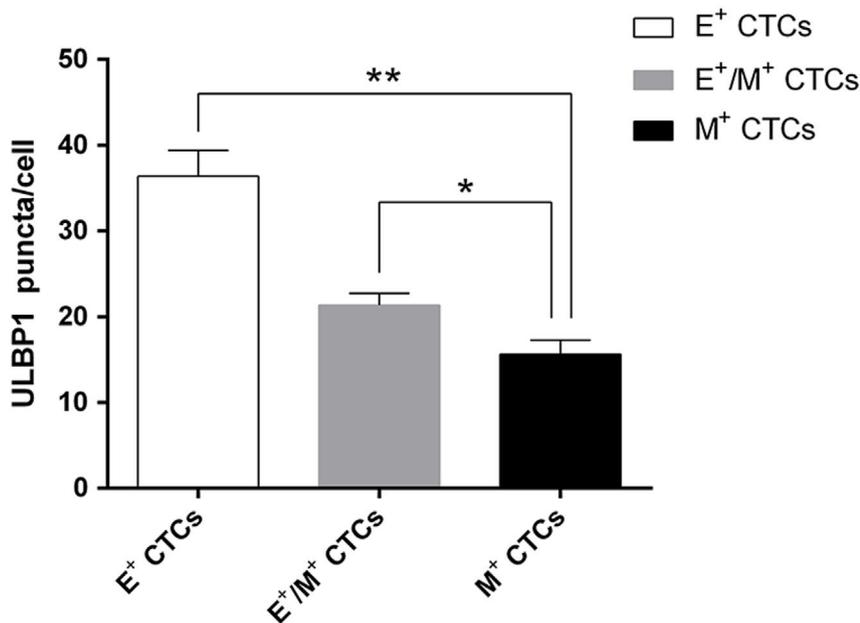
but not isolate CTCs directly from the millions of remaining cells, which resulted in the detection of nonhypotonic, damaged CTCs, regardless of the physical and biological properties of the CTCs. Meanwhile, to avoid false positive detection of CTCs, we identified CTCs with an immunofluorescence in situ hybridization (im-FISH) method based on chromosome 8 or 17 ploidy and CD45 expression. As shown in Figure 1, the CTCs exhibited negative membrane staining of CD45, and expressed multiple copies of chromosome 8 or 17 (≥3 green or yellow fluorescent dots), while the WBCs were identified as positive for CD45 (red fluorescence of the cell membrane) and exhibited diploid expression of chromosome 8 or 17 (two green or yellow fluorescence dots). Based on the enumeration of chromosome 8 or 17 and the expression of CD45, CTCs could

be easily differentiated from the thousands of other cells. Compared with a previous CTC detection strategy based on the phenotypic characteristics of the CTCs,[45,46] the method we used in this study was based on karyotyping of the CTCs, which facilitated straightforward identification of nonhematopoietic heteroploid CTCs, regardless of CTC phenotypic changes, such as downregulated or absent expression of EpCAM or other tumor cell surface markers.[47-49] Although the detection rate in our present study was not as high as the phenotypic methods reported previously, we believe that our methods achieved a higher accuracy rate.

Epithelial-mesenchymal transition has been established to involve the acquisition of a mesenchymal phenotype by epithelial cells. Studies from various research papers have

FIGURE 4 Epithelial-mesenchymal transition (EMT) induced by TGF-β1 in gastric SGC7901 cells. A, EpCAM and Vimentin expression in SGC7901 cells treated with TGF-β1 at concentrations of 5, 10, and 20 ng/mL for 72 h. B, EMT induced by TGF-β1 (20 ng/mL) in SGC7901 cells treated for 72 h. Scale bar = 50 μm. *P < .05, **P < .01
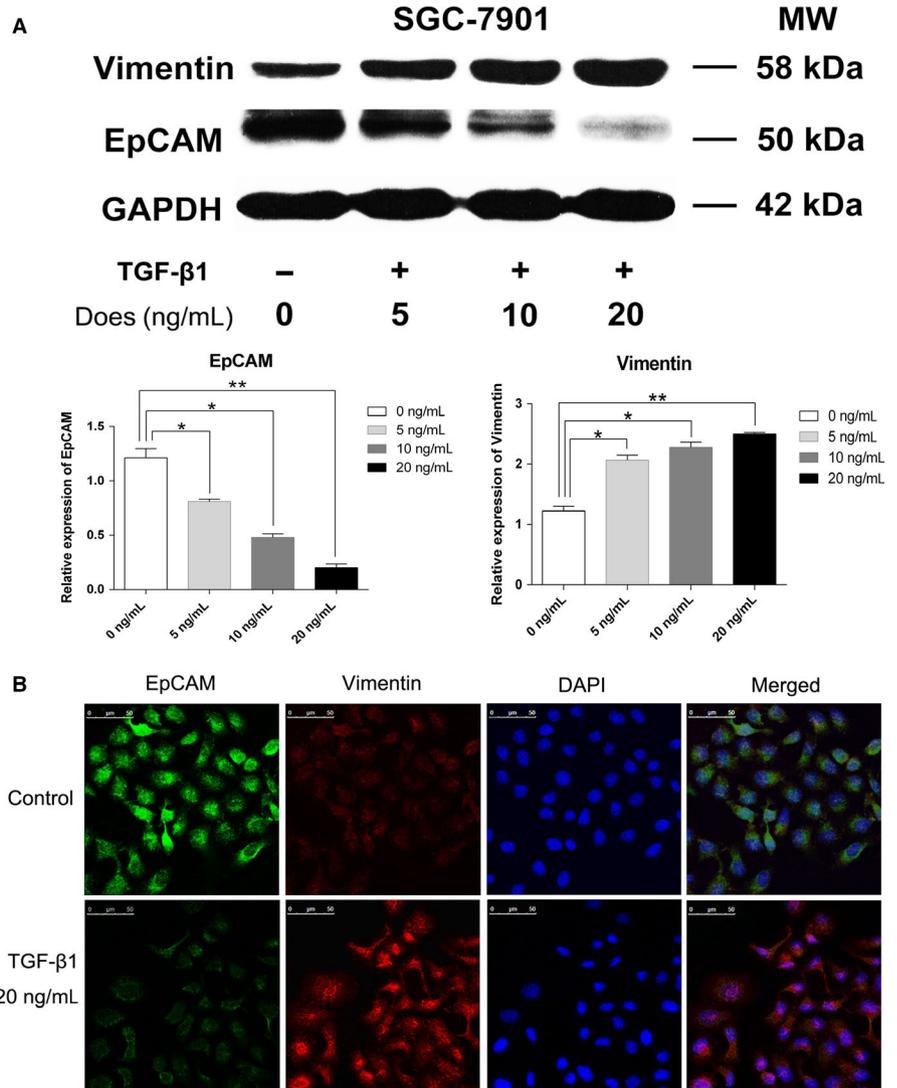
revealed that EMT is a commonly occurring phenomenon during the metastasis of CTCs in various cancers.[50-53] In this study, we detected three subtypes of CTCs, namely E⁺CTCs, E⁺/M⁺CTCs, and M⁺CTCs, and found that the positive rate and count range of each subpopulation differed in each CTC-positive patients. Additionally, the patients with more CTCs seemed to exhibit higher counts of E⁺/M⁺CTCs and M⁺CTCs. Researchers have documented that the phenotypic plasticity of CTCs is a bidirectional conversion among epithelial, mesenchymal, and hybrid epithelial/mesenchymal phenotypes, and that CTCs might successively undergo a process of EMT and MET in metastasis and the formation of secondary tumors.[54-56] Although the case number in our study was small, and we are unsure which subpopulation of CTCs resulted from EMT or MET, our results suggested that EMT may be a common process that CTCs must undergo in the metastasis of gastric cancer. In addition, we were unable to perform an effective statistical analysis on the relationship between CTC EMT phenotype and the clinicopathological features of patients due to

the small number of patients enrolled, and thus we intend to include more cases in a future study.

Immune escape and suppression are important phenomena and have long been proposed to constitute critical steps in both tumor formation and progression.[57-59] Theoretically, cancer cells that exit the primary tumor tissue, and thus leave the protection of the immunosuppressive tumor microenvironment, are more vulnerable to attack by immune effector cells.[60,61] Therefore, the survival of CTCs might be a vulnerable aspect of malignant tumor metastasis, and thus the mechanism enabling CTCs to evade immune effector cell killing may be an important target for prospective cancer therapy research. Studies have shown that tumor cells may shed or otherwise restrict the presentation of NK-cell receptor D (NKG2D) ligands, such as MICA, MICB, and ULBPs, involved in their recognition by NK cells or cytotoxic T lymphocytes (CTLs), or downregulate the expression of other factors that promote the activation of tumor-specific immune responses.[62-67] Furthermore, it is established that tumor cells that undergo EMT acquire phenotypic changes, involving the
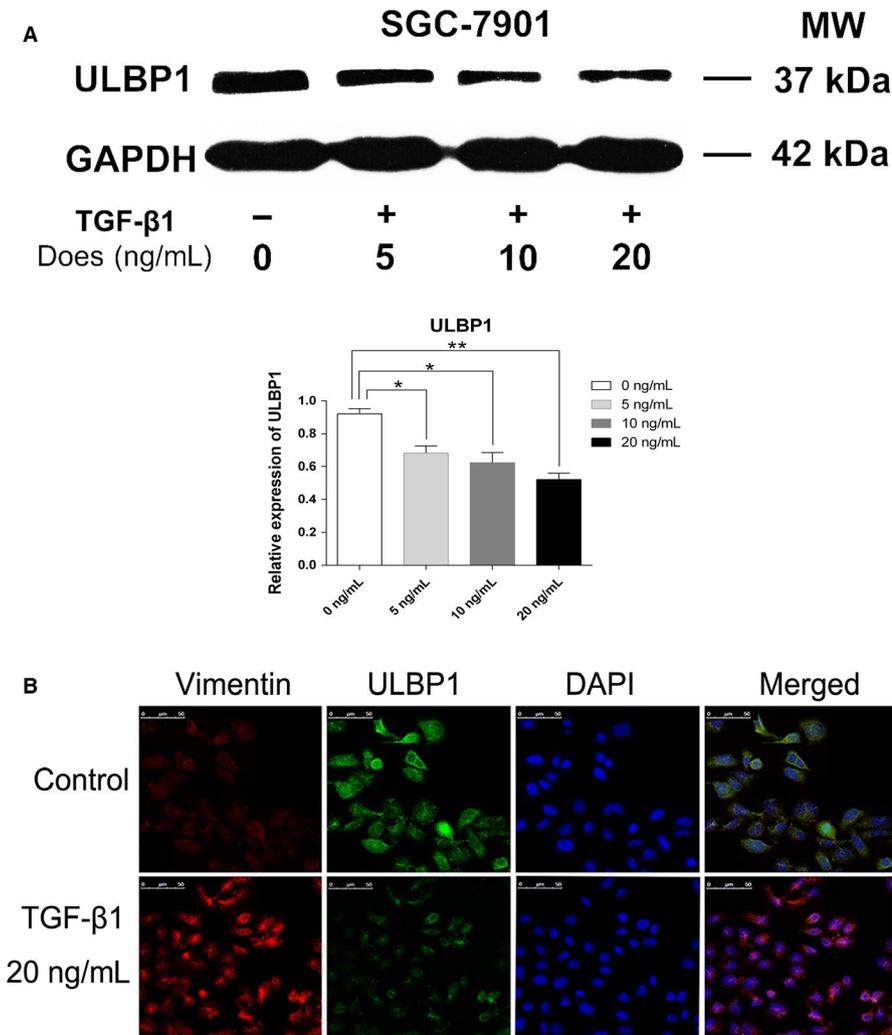
FIGURE 5 ULBP1 expression in gastric SGC7901 cells. A, ULBP1 protein expression in SGC7901 cells treated with TGF-β1 (5, 10, and 20 ng/mL) for 72 h. B, Epithelial-mesenchymal transition and expression of ULBP1 on SGC7901 cells treated with TGF-β1 (20 ng/mL) for 72 h. Scale bar = 50 μm. *P < .05, **P < .01

upregulation and downregulation of molecules. Increasing studies have also revealed that CTCs undergo EMT during the process of metastasis.[68-70] However, it remains unclear whether EMT of CTCs is accompanied by a change in ULBP1 expression. Therefore, the present study detected the cell-surface expression of EMT markers and ULBP1 on CTCs, and found that increased Vimentin and decreased EpCAM expression was accompanied by decreased ULBP1 expression. These results indicated that gastric CTCs might also shed or otherwise restrict the presentation of ULBP1 to escape immune effector killing. Moreover, the alteration of Vimentin and EpCAM expression on CTCs also indicated that EMT might be involved in the regulation of ULBP1 and in the immune evasion of CTCs. To build on previous studies, in which EMT induced a consistent upregulation of NKG2D ligand expression in colorectal carcinoma cell lines,[71] we performed this detection of EMT in CTCs. Recently, several studies also showed mesenchymal features in lung or breast cancer models was correlated with resistance to NK cells or cytotoxic T lymphocytes attacks.[72-75] However, to the best of our knowledge, this is the first study to evaluate the relationship between EMT and ULBP1 expression in gastric CTCs.

In addition, we acknowledge that other NKG2D ligands, such as ULBP2-6, MICA, and MICB, and other markers of EMT, such as E-cadherin, Twist, and Snail, might also be involved in the interaction of NK cells with tumor cells.[76,77] To further confirm the role of EMT, as well as the relationship between ULBP1 expression and EMT in the immune evasion of CTCs, functional studies introducing NK cells into a culture system of gastric cancer cell lines, and studies expanding the range of molecules detected on gastric CTCs, are now warranted.

To investigate the correlation between EMT and ULBP1, we successfully induced EMT in the gastric cancer cell line SGC7901 with TGF-β1 at concentrations of 5, 10, and 20 ng/mL in the current study, and the results showed that EMT markers, including EpCAM and Vimentin, as well as ULBP1, underwent significant changes in expression dependent on TGF-β1 dosage (Figures 4 and 5). Although the concentrations of TGF-β1 and the conditions of SGC7901 cell culture in vitro were not completely equivalent to the environment of gastric CTCs in the bloodstream, these results indicated a relationship between EMT and the immune evasion capacity of CTCs, and suggested a possible

mechanism regarding CTC immune evasion, where CTCs may shed or restrict the presentation of ULBP1. It has been established that TGF-β1 is not only a powerful extracellular EMT inducer, but also a crucial cytokine in the regulation of all aspects of the immune response, with a vital role in regulating responses mediated by almost all innate and adaptive immune cells.[78-80] Our results indirectly identified a potential role of TGF-β1 in the immune evasion of CTCs. Recently, platelets have been reported to promote and/or maintain the state of EMT in CTCs through platelet-secreted TGF-β.[81,82] Moreover, studies have reported that platelets could promote the survival of CTCs in the bloodstream by conferring resistance to the shear stress and to attack from NK cells.[83,84] However, it is unclear whether downregulation of ULBP1 via EMT-induced platelet-secreted TGF-β is a way in which platelets protect CTCs from immune effector killing. This may be vital in understanding the immune evasion capacity of CTCs and requires further study.

Overall, in this proof-of-principle study, we demonstrated that gastric CTCs in peripheral blood exhibited obvious EMT characteristics, as well as a downregulation of ULBP1, with TGF-β1-induced EMT accompanied by this downregulation of ULBP1 in vitro. Considering these results, we conclude that EMT of gastric CTCs might be involved in the immune evasion of CTCs through shedding or restricting the expression of ULBP1 during the process of metastasis. Further studies involving expanded NKG2D ligand expression on CTCs, its effect on immune evasion of CTCs and the possible regulation mechanism should be performed in the future to confirm this conclusion.

AUTHOR CONTRIBUTIONS
Baoguang Hu was involved in conceptualization and project administration. Xiaokun Tian was involved in manuscript writing. Yanbin Li and Chunyang Liu were involved in in vitro experiments. Yuming Li was involved in critical revision and final approval of the manuscript. Zhaodong Han, Tao Yang, and Jiajia An were involved in collection and analysis of clinical samples. Lingqun Kong was involved in collection of the clinical data.

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