Original contribution

EphB2 promotes cervical cancer progression by inducing epithelial-mesenchymal transition

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Summary EphB2, a receptor tyrosine kinase for ephrin ligands, is overexpressed in various cancers and plays an important role in tumor progression. However, the expression and functions of EphB2 in cervical cancer remain unknown. In this study, we performed immunohistochemistry in clinical cervical specimens and found that EphB2 was overexpressed in the cervical cancer specimens, and its expression correlated with cancer progression. The percentage of EphB2-positive cells increased gradually from 28% in the normal cervix to 40% in high-grade squamous intraepithelial lesions, and ultimately to 69.8% in squamous cell carcinomas (P < .05). We overexpressed EphB2 in HeLa cells and silenced EphB2 in cervical cancer (C33A) cells, which expressed low and high levels of EphB2, respectively. Exogenous EphB2 promoted cell migration, invasion, and an epithelial-mesenchymal transition (EMT) signature, which is a complex process that occurs during organogenesis and cancer metastasis, whereas EphB2 silencing had the opposite effect (P < .05). Furthermore, HeLa cells with exogenous EphB2 exhibited a stem cell–like state that promoted tumorsphere formation in vitro and exhibited tumorigenesis potential in vivo (P < .05), whereas EphB2 silencing in C33A cells inhibited these stem cell properties (P < .05). In addition, we investigated the intracellular signaling pathways in cervical cancer and found that R-Ras expression correlated positively with EphB2 in clinical samples, and its activity was regulated by EphB2 in cervical cancer. These findings demonstrate that EphB2 plays an important role in cervical cancer progression by orchestrating an EMT program through R-Ras activation.

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1. Introduction

Cervical cancer is the third most common cause of cancer-related mortality in women worldwide [1]. Epithelial-to-mesenchymal transition (EMT) of neoplasias, which plays a pivotal role in carcinogenesis and cancer progression, is an important factor in mortality risk [2,3]. This transition is characterized by a loss of epithelial characteristics (ie, cell-cell contacts and apical-basal polarity) and the concomitant acquisition of mesenchymal characteristics, which enable cancer cells to spread more easily and thus be more invasive [4]. This conversion is demonstrated by down-regulation of epithelial markers and up-regulation of mesenchymal markers [4].

Erythropoietin-producing human hepatocellular carcinoma (Eph) receptors constitute the largest family of receptor tyrosine kinases in the human genome [5]. When ligands bind to Eph receptors, the kinase domain becomes phosphorylated, resulting in the activation of signaling cascades such as ephexin, Src, Nck, Crk, and R-Ras [6]. EphB2 overexpression has been reported in a variety of human cancers. Previous studies have shown that EphB2 plays important roles in colon cancer development [7], and EphB2 phosphorylation promotes glioma cell migration and invasion by eliciting signaling through R-Ras [8]. In addition, EphB2 was observed at the base of the colonic crypt in colorectal cancer, demonstrating that EphB2 is expressed in colon progenitor cells [9,10].

To date, the expression pattern and mechanism of EphB2 in cervical cancer remain unknown, although considerable efforts have been made to elucidate the biological functions of the Eph receptor in many cancers. In this study, we found that EphB2 was up-regulated in cervical cancer and induced cervical cancer cells to undergo EMT and acquire stem cell properties via activation of the R-Ras pathway, demonstrating that EphB2 plays an important role in carcinogenesis and cancer progression.

2. Materials and methods

2.1. Cell lines and clinical samples

Cervical cancer cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD) and cultured in standard conditions. Clinical samples were obtained from patients undergoing surgical resection in The Second Affiliated Hospital of Xi’an Jiaotong University (China) after they gave informed consent. Samples included 25 normal cervical (NC) tissues, 20 high-grade squamous intraepithelial lesions (HSILs), and 53 cervical squamous cell carcinoma (SCC) samples. Hematoxylin and eosin–stained sections were used to demonstrate pathological diagnosis. Clinical stages were identified according to the International Federation of Gynecology and Obstetrics classification system [11].

2.2. Immunohistochemistry and immunocytochemistry

Formalin-fixed, paraffin-embedded cervical cancer tissues were subjected to the Catalyzed Signal Amplification System (Boster, Wuhang, China), according to the manufacturer’s instructions. Sections were incubated with primary antibody after dewaxing and rehydration and then incubated with biotinylated secondary antibody and visualized with 3,3′-diaminobenzidine; the nuclei were counterstained with hematoxylin. For immunocytochemistry (ICC), cells seeded on polylysine-coated glass slides were fixed in 4% paraformaldehyde and processed as tissue sections. Negative controls were prepared using the same procedure, but phosphate-buffered saline was substituted for primary antibody.

To determine immunoreactivity score (IRS) of EphB2 or R-Ras, 3 independent researchers examined the sections in 10 representative microscopic fields. The results were determined by multiplying the staining intensity (scored as 0, no staining; 1, weak staining; 2, moderate staining; or 3, strong staining) by the percentage of positive cells (scored as 1, 0-25% positive cells; 2, 26%-74% positive cells; 3, 75%-89% positive cells; or 4, 90%-100% positive cells). Positive expression was considered IRSpre.

2.3. Western blot analysis

Cells were lysed in radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail (Complete Mini; Roche Diagnostics, Branchburg, NJ). Proteins were separated by sodium dodecyl polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Then membranes were probed with specific primary antibodies, followed by incubation with horseradish peroxidase–conjugated secondary antibodies (Thermo Fisher Scientific Inc, New York, NY). Bands were visualized using Chemiluminescent HRP Substrates (Millipore, Billerica, MA).

2.4. RNA isolation and real-time polymerase chain reaction

Total RNA was isolated using the TRIzol Reagent (Invitrogen, Carlsbad, CA), and first-strand complementary DNA (cDNA) was synthesized using M-MuLV reverse transcriptase and random hexamer primers (MBI, Hanover, MD), according to the manufacturer’s instructions. The resulting cDNA was used for real-time polymerase chain reaction (PCR) using the SYBR Green PCR Kit (Takara, Japan), and data were normalized to the GAPDH housekeeping gene. Real-time PCR and data collection were performed on a Bio-rad IQ5 real-time PCR instrument.

3. Discussion

The present study demonstrated that EphB2 is expressed in cervical cancer tissues and that its expression is associated with clinicopathological parameters. These findings suggest that EphB2 plays a role in cervical cancer progression and may serve as a potential therapeutic target.
2.5. Plasmid construction and stable transfectants

The human EphB2 CDS fragment was PCR amplified from C33A cells and cloned into pIRES2-EGFP vector (Clontech, Mountain View, CA). EphB2 shRNA vectors were designed and purchased from GenePharm Company (GenePharm, Shanghai, China). To generate stably transfected cell lines, cells were transfected with vectors (pIRES2-EGFP, pIRES2-EGFP-EphB2, pGPU6/GFP-scramble, or pGPU6/GFP-ShEphB2) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocols, then selected by G418 reagent (Calbiochem, La Jolla, CA). Individual drug-resistant clones were identified by Western blotting.

2.6. Migration and invasion assays

Transwell chambers (Corning Costar, Corning, NY) coated with/without matrigel (BD Biosciences, Hercules, CA) were used for the invasion/migration assay. Cells (1-5 × 10^5) were plated in the upper chamber of the transwells and allowed to invade across the membranes for 24 to 48 hours, then invading cells were fixed and stained with hexamethylandamin pararosaniline. The number of penetrated cells was determined in 10 representative microscopic fields.

2.7. Tumorsphere formation assay

To obtain tumorspheres, 10^3 cells per well were cultured in DMEM/F12 with B-27 (Invitrogen), epidermal growth factor, and basic fibroblastic growth factor (PeproTech, Rocky Hill, NJ). Under these culture conditions, only cancer stem cells, early progenitor cells, and undifferentiated cancer cells could survive and proliferate[12]. The formed tumorspheres were examined and counted under a microscope.

2.8. Tumor xenotransplantation

Six- to 8-week-old female BALB/c mice were injected subcutaneously with 1 to 5 × 10^4 cells. The development and progression of solid tumors were monitored longitudinally for 12 weeks. Tumor volume (V) was determined by length (a) and width (b) according to the formula V = ab^2/2. Mice were euthanized when they met the institutional euthanasia criteria for tumor size and overall health conditions. The experimental protocols were evaluated and approved by the Animal Care and Use Committee of the Medical School of Xi’an Jiaotong University.

2.9. Immunoprecipitation

For immunoprecipitation (IP), cells were lysed in a Nonidet P-40 buffer, and equivalent amounts of protein (200 μg) were precleared and immunoprecipitated from lysates and washed with lysis buffer, as previously described [8]. The immunoprecipitates were analyzed by Western blotting.

2.10. Statistical analyses

Each sample was assayed in triplicate, and each experiment was repeated 3 times. Spearman rank correlation test, t test, and log-rank test were performed by the Statistical Package of Social Science (SPSS) 16.0 (SPSS, Chicago, IL). Data are presented as mean ± SD. P values less than .05 were considered statistically significant.

3. Results

3.1. EphB2 is up-regulated in cervical cancer

To evaluate the expression of EphB2 in cervical cancer specimens, we examined tissue samples from the NC, HSILs, and SCCs by immunohistochemistry (IHC). EphB2 was expressed in most of the cervical cancer cells but was only expressed in basal cells of the NC epithelia, and all positive staining was on the cell membrane and cytoplasm (Fig. 1A). In addition, the EphB2 score was higher for HSILs and SCCs compared with the NC (Fig. 1B, P < .05). Specifically, the number of EphB2-positive cells gradually increased from 28% (7/25) in the NC to 40% (8/20) in HSILs, and ultimately to 69.8% (37/53) in SCCs (Fig. 1C, P < .05).

Further investigation of EphB2 and clinical disease progression showed that EphB2 expression was higher in advanced stage II to III cancers (86.5%; 32/37) than in stage I cancer (25%; 5/16) (Table, P < .05). Otherwise, EphB2 overexpression significantly correlated with tumor progression and stage malignancy; however, its overexpression was not associated with other clinicopathological parameters (Table).

3.2. EphB2 is highly expressed in metastatic cervical cancer cell lines

We further investigated the EphB2 levels in 5 cervical cell lines, and found that higher EphB2 expression in metastatic cell lines (HT-3, Caski) was comparable with nonmetastatic cell lines (HeLa, SiHa) (Fig. 2A and B). Real-time PCR showed that EphB2 RNA levels correlated with EphB2 protein levels (Fig. 2C).

3.3. EphB2 Induces EMT in cervical cancer cells

To investigate whether EphB2 regulates invasion and metastasis in cervical cancer, we stably overexpressed EphB2 in HeLa cells or silenced EphB2 in C33A cells, which resulted in low and high levels of endogenous EphB2 expression, respectively, as confirmed by Western blotting (Fig. 3A). Cells with exogenous EphB2 displayed an elongated fibroblast-like morphology with loss of cell-to-cell contact, whereas control cells retained their morphology.
with tight cell-cell adhesions (Fig. 3B). These findings showed that EphB2 overexpression induced HeLa cells to undergo EMT-like phenotypic changes.

We further examined the effects of EphB2 on the epithelial and mesenchymal characteristics of cervical cancer cells and found that HeLa cells overexpressing EphB2 showed down-regulation of E-cadherin and up-regulation of vimentin (Fig. 3B and C). In contrast, EphB2 silencing in C33A cells led to up-regulation of E-cadherin expression, although vimentin was not down-regulated to its low endogenous levels in this cell line (Fig. 3C).

Real-time PCR demonstrated that expression of ectopic EphB2 led to several molecular features of EMT, including up-regulation of the mesenchymal markers, CDH2 and Fibronectin, as well as key transcriptional inducers such as

**Table**  
Correlation between EphB2 expression and clinicopathologic parameters in patients with cervical cancer

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. of patients</th>
<th>EphB2 IRS Positive</th>
<th>%</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Age (y)</td>
<td></td>
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</tr>
<tr>
<td>&lt;45</td>
<td>18</td>
<td>12</td>
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<tr>
<td>≥45</td>
<td>35</td>
<td>25</td>
<td>71.4286</td>
<td>&gt;.05</td>
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<tr>
<td>Grade</td>
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<td></td>
</tr>
<tr>
<td>I</td>
<td>7</td>
<td>5</td>
<td>71.4286</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>II</td>
<td>19</td>
<td>13</td>
<td>68.4211</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>III</td>
<td>27</td>
<td>19</td>
<td>70.3704</td>
<td>&lt;.05</td>
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<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>16</td>
<td>5</td>
<td>31.25</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>II-III</td>
<td>37</td>
<td>32</td>
<td>86.4865</td>
<td>&lt;.05</td>
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</tbody>
</table>
Snail1, Snail2, Twist2 (Fig. 3D). Silencing of EphB2 in C33A cells had the opposite effect (Fig. 3E). Ectopic EphB2 expression in HeLa cells caused a 2-fold increase in migratory and invasive behavior compared with control cells (Fig. 3F, \( P < .05 \)), whereas EphB2 silencing in C33A cells decreased those behaviors to three-fourths those of the controls (Fig. 3F, \( P < .05 \)).

3.4. EphB2 induces cervical cancer cells to exhibit a stem cell state

Previous studies have shown that cells undergoing EMT acquire stem cell phenotypes [13]. To determine whether EphB2 induces stem cell phenotypes upon EMT, we carried out tumorsphere formation and tumor xenotransplantation. Overexpression of EphB2 in HeLa cells increased both the size and number of tumorspheres compared with the control, whereas silencing of EphB2 inhibited tumorsphere formation in C33A cells (Fig. 4A). As shown in Fig. 4B, EphB2 elevated the percentage of formed tumorspheres in HeLa cells from 12% ± 4.32% to 36.5% ± 7.32% and decreased the percentage of formed tumorspheres in C33A cells with EphB2 silencing from 94.75% ± 22.7% to 49% ± 17.6% (Fig. 4B, \( P < .05 \)). Elevated levels of EphB2 remarkably increased the frequency of tumor formation and reduced the latency period in HeLa cells (Fig. 4C, \( P < .05 \)), and silencing of EphB2 had the opposite effect (Fig. 4D, \( P < .05 \)). After tumor initiation, EphB2 had no significant effects on xenograft growth rates (Fig. 4E and F, \( P > .05 \)).

3.5. EphB2/R-Ras signaling is activated in cervical cancer

Although previous studies have indicated that the EphB2/R-Ras signaling pathway is activated in some cancers [8,14], the functional relationship between EphB2 and R-Ras in cervical cancer remains unknown. To explore whether the EphB2/R-Ras intracellular signaling pathway activated in cervical cancer, we examined R-Ras expression in clinical tissue samples by IHC and found that R-Ras expression was mainly localized to the cell membrane and cytoplasm. Similar to EphB2, R-Ras was expressed in most of the cervical cancer cells, but only in basal cells of the NC epithelia (Fig. 5A). The IRS of R-Ras was dramatically elevated in HSILs and SCCs compared with the NC (Fig. 5B, \( P < .05 \)). Importantly, the expression levels of EphB2 and R-Ras directly correlated in these samples (\( r = 0.5358; \) Fig. 5C, \( P < .05 \)), indicating that, to a large extent, the 2 proteins were concurrently expressed in cervical cancer.

Moreover, we examined the expression of R-Ras in cervical cancer cell lines by ICC (Fig. 5D) and found that R-Ras was highly expressed on the cell membrane and in all 5

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**Fig. 2** Expression of EphB2 in cervical cancer cell lines. A, ICC for the EphB2 protein and negative control (×1000) in HeLa, SiHa, C33A, HT-3, and Caski cells. B and C, Western blot (B) and quantitative reverse-transcription PCR (C) show different levels of EphB2 protein and mRNA expression in cervical cancer cells.
Fig. 3  EphB2 induces an EMT program in cervical cancer cells. A, Western blot analysis was used to characterize the overexpression and silencing of EphB2 in HeLa and C33A cells, respectively. B, Phase contrast images (∼200) and ICC images (∼1000) of H/Ctr and H/Eph cells show the morphology and staining of E-cadherin and vimentin. C, Western blot analysis showing expression of E-cadherin and vimentin. D and E, Quantitative reverse-transcription PCR of the expression of EMT-related genes in HeLa (D) and C33A (E). Bar charts represent fold expression changes in cells with overexpressed/silenced EphB2 compared with control cells. F and G, The charts are shown for the transwell migration (F) and invasion (G) assays in HeLa and C33A cells. Data are shown as means ± SD. *P < .05 Ctr and Scr are abbreviated for control and scramble, respectively.
cell lines (HeLa, SiHa, C33A, HT-3, Caski). To further determine whether R-Ras is activated by EphB2 in cervical cancer, we examined the phosphorylation level of R-Ras in HeLa cells overexpressing EphB2. IP of phosphotyrosine revealed that exogenous EphB2 elevated R-Ras tyrosine phosphorylation levels in HeLa cells compared with control cells (Fig. 5E). Silencing of EphB2 in C33A cells reduced the phosphorylation levels of R-Ras (Fig. 5E). Together, these data demonstrate that EphB2/R-Ras signaling plays an important role in cervical cancer.

4. Discussion

Increasing evidence suggests that EphB2 overexpression is associated with several human cancers and, specifically, is important for cancer metastasis [8,15]. A previous cDNA microarray analysis study showed elevated expression of EphB2 in cervical cancer [16]. In this study, we found that EphB2 was up-regulated in cervical cancer and, during cancer progression, the percentage of EphB2-positive cells increased (Fig. 1). These results point to the involvement of EphB2 in cervical cancer development. The higher expression of EphB2 observed in metastatic cell lines (Fig. 2) also suggests that EphB2 may play a role in malignant transformation and metastasis.

The EMT is a crucial step in cancer invasion and metastasis [2,17]. Several signaling pathways that are important for normal and cancer development, including the TGF-β, Wnt, Notch, epidermal growth factor, and FGF pathways, have been implicated in governing EMT. These pathways affect EMT by regulating the expression of crucial EMT-related proteins.

**Fig. 4** EphB2 induces cervical cancer cells to exhibit a stem cell state. A, Phase contrast images (×200) show the tumorsphere formation assays in HeLa and C33A cells. B, Bar chart illustrates the quantitative analysis of the tumorspheres. Data are shown as mean ± SD. C-F, The charts are shown for the tumor-free survival probability of HeLa (C) and C33A (D) and tumor volumes for HeLa (E) and C33A (F) over time for BALB/c mice with subcutaneous injection. *P < .05.
Fig. 5  EphB2/R-Ras signaling is activated in cervical cancers. A, IHC for R-Ras protein and the negative control (×1000) in paraffin-embedded sections of NC tissues, HSILs, and SCCs. B, Box chart is shown for R-Ras expression in each group of clinical cervical tissues. *P < .05. C, Direct correlation between R-Ras IRS and EphB2 IRS in clinical cervical tissues (r = 0.632; P < .05). D, IHC for the R-Ras protein and negative control (×1000) in HeLa, SiHa, C33A, HT-3, and Caski cells. E, HeLa cells with EphB2 overexpression and C33A cells with EphB2 silencing were subjected to IP with anti–R-Ras antibody. The immunoprecipitates were probed by immunoblotting with phosphotyrosine, and inputs were probed for R-Ras.
transcription factors, including the Snail, ZEB, and Twist family proteins. Transcription factors, like Twist and Snail, are known to be associated with cellular EMT, promoting cadherin switching and vimentin expression [4,18].

Several studies examining the role of EMT progression in cervical cancer have provided evidence of Twist2, E-cadherin, and vimentin regulating EMT during tumorigenesis and cancer progression [19]. In addition, NF-κB, p16\(^{\text{INK4a}}\), hypoxia, and miR155 were also reported to participate in EMT in cervical cancer [20-23]. In this study, we found that EphB2 functioned as an EMT trigger and activated the EMT program in cervical cancer epithelial cells by repressing the epithelial phenotype, inducing the mesenchymal phenotype, and dramatically increasing its migration and invasion abilities. Silencing of EphB2 expression in C33A cells inhibited EMT progression (Fig. 3).

Recent studies have shown that cells undergoing EMT acquire stem cell phenotypes consistently [13,24]. Cancer-initiating cells from 4 well-known human cervical–derived cell lines express markers characteristic of stem cells and EMT [25]. In this study, we found that EphB2 was abundant on the surface of cervical cancer cells but was located mainly on the basal layer of NCs (Fig. 1), which is considered to be a reserve cell layer, presumed to contain the cervical stem cells. Furthermore, we observed that ectopic EphB2 was able to increase the tumoursphere-forming ability and tumorigenesis potential, while silencing of EphB2 decreased the stem cell properties (Fig. 4). Thus, we hypothesized that EphB2 is a potential stem cell marker of cervical cancer.

Previous research has demonstrated that Ras promotes cancer aggressiveness by inducing EMT in several cancers [26-28] and Ras/MAPK signaling regulates the EMT program in cooperation with PTEN, TGF-β, and other factors [26,29]. It has been shown that the EphB2/R-Ras signaling pathway is activated in glioma cells and mediates migration and invasion [8]. In this study, we found that R-Ras expression was up-regulated in cervical cancer and correlated with EphB2 expression, providing evidence of EphB2/R-Ras signaling in cervical cancer. In addition, phosphorylation of R-Ras was regulated by EphB2 in cervical cancer, indicating that R-Ras works downstream of EphB2 and contributes to cervical cancer migration and invasion.

In conclusion, we uncovered an important function for EphB2 in the progression of cervical cancer wherein it orchestrates an EMT program and contributes to stem cell–like properties. In addition, EphB2/R-Ras signaling was activated by EphB2 in cervical cancer cells. These results indicated that EphB2 may be a marker of EMT and cancer stem cells and further suggest that EphB2 may be a potential target for therapeutic intervention to inhibit cervical cancer progression.

References


