Overexpression of ephrinB2 and EphB4 in tumor advancement of uterine endometrial cancers

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Background: The ligand ephrinB2 and the corresponding receptor EphB4 contribute to tumor growth in various human tumors. This prompted us to study the expression and localization of ephrinB2 and EphB4 in uterine endometrial cancers to analyze the ephrinB2/EphB4 functions against clinical backgrounds.

Materials and methods: We carried out immunohistochemistry and real-time RT-PCR to determine the histoscores and messenger RNA (mRNA) levels of ephrinB2 and EphB4, respectively, in 68 uterine endometrial cancers and 16 normal endometrium tissue samples. Patient prognoses were analyzed with a 60-month survival rate.

Results: The localization of ephrinB2 and EphB4 was dominantly in the cancer cells of uterine endometrial cancer of all cases given. EphrinB2 and EphB4 histoscores were highly correlated with ephrinB2 and EphB4 mRNA levels, respectively ($r = 0.864$ and $r = 0.615$, $P < 0.01$). Both the histoscores and mRNA levels of ephrinB2 and EphB4 significantly increased with clinical stages ($I < II < III$, $P < 0.01$), dedifferentiation ($G_1 < G_2 < G_3$, $P < 0.01$) and myometrial invasion ($A < B < C$, $P < 0.01$ for ephrinB2 and $P < 0.05$ for EphB4) in uterine endometrial cancers. The 60-month survival rates of the 34 patients with high ephrinB2 and EphB4 expression were poor (59% and 62%, respectively), while for the other 34 patients with low ephrinB2 and EphB4 expression, they were significantly higher (85% and 82%, respectively).

Conclusions: EphrinB2 and EphB4 were overexpressed during the tumor advancement as dedifferentiation and myometrial invasion. Therefore, ephrinB2/EphB4 might work on tumor advancement and may be recognized as a novel prognostic indicator for uterine endometrial cancers.

Key words: EphB4, ephrinB2, prognostic indicator, tumor advancement, uterine endometrial cancers

Introduction

The Eph family of receptor tyrosine kinase is the largest among the receptor tyrosine kinase superfamilies, containing 14 distinct receptors, for which at least eight ligands have been identified [1, 2]. On the basis of sequence homology and ligand affinity, the Eph receptors can be divided into two subclasses, EphA (A1–A8), which are anchored on plasma membranes through a glycosylphosphatidylinositol linkage and bound by their ligand ephrinA (A1–A5), whereas EphB (B1–B6) are bound by ephrinB (B1–B3) and tethered to the membrane by a transmembrane domain [2], with the exception of EphA4, which can bind with A-type and most B-type ligands.

To date, various biological functions have been attributed to ligand ephrins and the corresponding receptor Ephs, including vascular development, tissue border formation, cell migration, axon guidance and synaptic plasticity [3–6]. As both ephrins and Ephs are membrane bound and therefore binding and activation of ephrins and Ephs require cell-to-cell interaction rather than long-range communication, they mediate bidirectional signaling cascades [7]. EphB4 is known to interact only with ephrinB2 among all ephrinB ligands [8–11]. The genes for Eph, located at chromosome 7q22.1 and initially isolated from a human hepatocellular carcinoma cell line Hep3B [12], and ephrin have been recognized to be differentially expressed in various human tumors, such as malignant melanoma, neuroblastoma and cancers of the prostate, breast, lungs, esophagus, gastrium and colorectum [13–18]. This prompted us to study the expression and localization of ligand ephrinB2 and receptor EphB4 in uterine endometrial cancers to analyze the ephrinB2/EphB4 functions against clinical backgrounds.

Materials and methods

Patients and Tissues

Prior informed consent for the following studies was obtained from all patients and approval was given by the Research Committee for Human Subjects, Gifu University School of Medicine. Sixty-eight patients ranging from 35 to 79 years of age with uterine endometrial cancers (23 stage I cases, 21 stage II cases and 22 stage III cases; and 24 well-differentiated, 22 moderately differentiated and 22 poorly differentiated endometrioid...
adenoacarcinoma cases) underwent curative resection, which produced macroscopically disease-free status, as shown in Table 1, and 16 patients ranging from 35 to 48 years of age with a regular menstrual cycle underwent hysterectomy for uterine leiomyoma with histologically normal uterine endometrium at the Department of Obstetrics and Gynecology, Gifu University School of Medicine, from September 1995 to March 2001. None of the patients had received any preoperative therapy. The tissues of uterine endometrial cancer and normal uterine endometrium were obtained immediately after surgery. The tissues for RNA isolation were snap frozen and stored at −80°C, and those for immunohistochemistry were fixed with 10% formalin and embedded in paraffin wax. The clinical backgrounds of uterine endometrial cancer were evaluated by International Federation of Gynecology and Obstetrics classification [19].

**immunohistochemistry**

Four-microcentimeter sections of formalin-fixed, paraffin-embedded tissue samples from uterine endometrial cancers were cut with a microtome and dried overnight at 37°C on a silated-slide (Dako, Carpintiera, CA, USA). The protocol of universal Dako Labelled Streptavidin–Biotin kit (Dako) was followed for each sample. Samples were deparaffinized in xylene at room temperature for 30 min, rehydrated with graded ethanol and washed in phosphate-buffered saline (PBS). The samples were then placed in 10 mM phosphate-buffered saline (PBS). The samples were then placed in 10 mM sodium phosphate buffer (pH 6.0) and boiled in a microwave for 10 min for epitope retrieval. Endogenous peroxidase activity was quenched by incubating tissue sections in 3% H2O2 for 10 min. The primary antibodies rabbit ephrinB2 (sc-1010, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit EphB4 (sc-5536, Santa Cruz) were used overnight at 4°C at dilutions of 1:75 and 1:100, respectively. The slides were washed and biotinylated anti-rabbit secondary antibody (Dako) was applied for 30 min. After rinsing in PBS, streptavidin-conjugated horseradish peroxidase (Dako) was added for 30 min. Slides were then washed and treated with the chromogen 3,3′-diaminobenzidine (Dako) for 5 min, then rinsed in PBS, counterstained with Mayer’s hematoxylin, dehydrated in graded ethanol, cleared in xylene and coverslipped with a mounting medium, Entellan New (Merck, Darmstadt, Germany). Rabbit preimmune animal serum (Dako) was used for negative controls instead of the primary antibody for ephrinB2 or EphB4.

**assessment of histochemical score (histoscore)**

All sections of immunohistochemical staining for ephrinB2 and EphB4 were evaluated in a semiquantitative fashion according to the method described by McCarty et al. [20], which considers both the intensity and the percentage of cells stained at each intensity. Intensities were classified as 0 (no staining), 1 (weak staining), 2 (distinct staining), 3 (strong staining) and 4 (very strong staining). For each stained section, a value–designated histoscore was obtained by applying of the following algorithm: histoscore = Σ(i + 1) × Pi, where i and Pi represent intensity and percentage of cells that stain at each intensity, respectively, and the corresponding histoscores were calculated separately.

**preparation of standard template for real-time RT-PCR**

Internal standard template for real-time PCR was produced by PCR amplification using the primers for ephrinB2 gene, 570-989 in the complementary DNA (cDNA) (ephrinB2-TS: 5′-GTCCAGAAGTACGAATGGTG-3′ and ephrinB2-TAS: 5′-GTATCATGCTCAGGAGATGTC-3′), and for EphB4 gene, 2201-2625 in the cDNA (EphB4-TS: 5′-AGATGATCTCAGCATG-3′ and EphB4-TAS: 5′-GATGCTGGAGGAGAACCTT-3′). The DNA template was purified using a GeneClean II kit (Qbiogene, Irvine, CA, USA). The copy numbers of the standard template were determined to quantify ephrinB2 and EphB4 messenger RNA (mRNA) levels in samples for real-time RT-PCR.

**real-time RT-PCR**

Total RNA was extracted with the acid guanidinium thiocyanate-phenol-chloroform method [21]. The total RNA (3 µg) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (MMLV-RT, 200 U/µl Invitrogen, Carlsbad, CA, USA) and the following reagents: 250 mM Tris–HCl (pH 8.3), 375 mM KCl, 15 mM MgCl2, 0.1 M diethiothreitol, 10 mM deoxycytidine (deoxyadenosine, deoxythymidine, deoxyguanosine and deoxyctydine) triphosphates (dNTPs) mixture and random hexamers (Invitrogen) at 37°C for 1 h. The reaction mixture was heated for 5 min at 94°C to inactivate MMLV-RT.

Real-time PCR was carried out with a Takara Ex Taq R-PCR kit, version 1.0 (Takara, Otsu, Japan), using a smart cycling system (Cepheid, Sunnyvale, CA, USA). The reaction solution (25 µl) contained Takara Ex Taq HS (5 units/µl), 10× R-PCR Buffer, 250 mM Mg2+ solution, 10 mM dNTP mixture, SYBR Green 1 (1 : 1000 dilution;Cambrex Bio Science Rockland, Inc. Rockland, ME, USA) and 20 µM of the primers for ephrinB2 gene, 679-785 in the cDNA (ephrinB2-S: 5′-GGAACATCCCTGTGTTGCAAG-3′ and ephrinB2-AS: 5′-CCTCTTGCAGAACATCGGA-3′) and for EphB4 gene, 2501-2625 in the cDNA (EphB4-S: 5′-ACGCACTGACGATGGACTG-3′ and EphB4-AS: 5′-GCCACATGCTAGTCAACAG-3′) with the transcribed total RNA from the tissue and a serially diluted standard template. The real-time PCRs were initially denatured by heating at 95°C for 30 s, followed by 40 cycles consisting of denaturation at 94°C for 10 s, annealing at 55°C for 5 s and extension at 72°C for 20 s. A strong linear relationship between the threshold cycle and the log concentration of the starting DNA copy number was always shown (correlation coefficient >0.99). Quantitative analysis was carried out to determine the copy numbers of each sample.

**statistical analysis**

EphrinB2 and EphB4 mRNA levels were determined from three parts taken from each tumor, and each sample was analyzed in triplicate. The differences in the histoscores and mRNA levels of ephrinB2 and EphB4 were analyzed by Student’s t-test. The correlation coefficients were evaluated both by linear regression analysis and bivariate Pearson correlation. Survival rates were calculated using the Kaplan–Meier method and analyzed by the log-rank test. Differences were considered significant when P was <0.05.

**results**

EphrinB2 and EphB4 localization by immunohistochemistry

All the uterine endometrial cancer specimens revealed strong staining for ephrinB2 and EphB4 in the cancer cells and very

| Table 1. Clinical background of uterine endometrial cancer patients (n = 68) |
|---|---|---|---|---|
| Clinical background | Clinical stage | Histological grade | Myometrial invasion |
| | I | II | III | G1 | G2 | G3 | A | B | C |
| Number of patients | 25 | 21 | 22 | 24 | 22 | 22 | 23 | 21 | 24 |

Stage I is carcinoma confined to the uterine corpus; stage II involves the corpus and the cervix, but has not extended outside the uterus; stage III extends outside of the uterus but is confined to the true pelvis; G1, well-differentiated adenocarcinoma; G2, moderately differentiated adenocarcinoma; G3, poorly differentiated adenocarcinoma, A, tumor limited to the endometrium; B, invasion to less than half the myometrium; C, invasion to more than half the myometrium.
faint staining in vascular endothelial cells. Positive staining for ephrinB2 and EphB4 was in the cytoplasm and cell membrane of cancer cells. Immunohistochemical staining for ephrinB2 and EphB4 of a representative case of well-differentiated endometrioid adenocarcinoma of the uterine endometrium is shown in Figure 1.

**Histoscores and mRNA levels of ephrinB2 and EphB4 according to clinical backgrounds**

EphrinB2 histoscores of cancer cells significantly correlated with ephrinB2 mRNA levels \( (r = 0.864, P < 0.01) \), while EphB4 histoscores of cancer cells correlated with EphB4 mRNA levels \( (r = 0.615, P < 0.01) \). There were significant differences in the histoscores and mRNA levels of ephrinB2 and EphB4 between normal uterine endometria and uterine endometrial cancers classified into clinical stages, histological grades and myometrial invasion.

Both the histoscores and mRNA levels of ephrinB2 and EphB4 significantly increased according to clinical stage \( (I < III < III, P < 0.01) \) as shown in Figure 2A; according to histological grade \( (G1 < G2 < G3, P < 0.01) \) as shown in Figure 2B and according to depth of myometrial invasion \( (A < B < C, P < 0.01 \) for ephrinB2 and \( P < 0.05 \) for EphB4) as shown in Figure 2C.

**Correlation between ephrinB2 and EphB4 with patients’ overall survival**

We analyzed the prognosis for the 68 patients who underwent curative resection. The values of 260 in ephrinB2 histoscore and \( 6.8 \times 10^6 \) DNA copies/µg total RNA in ephrinB2 mRNA level and 190 in EphB4 histoscore and \( 1.9 \times 10^5 \) DNA copies/µg total RNA in EphB4 mRNA level were the median values, and adopted to divide the 68 patients into two groups of 34 patients each.

The 60-month survival rate of the 34 patients with high ephrinB2 (cases with ephrinB2 histoscore >260; the same as those with ephrinB2 mRNA levels \( >6.8 \times 10^6 \) DNA copies/µg total RNA) was 59%, while that of the other 34 patients with low ephrinB2 (cases with ephrinB2 histoscore <260; with ephrinB2 mRNA levels \( <6.8 \times 10^6 \) DNA copies/µg total RNA) was 83%. The survival rate of the 34 patients with high EphB4 (cases with EphB4 histoscore >190; the same as those with EphB4 mRNA levels \( >1.9 \times 10^5 \) DNA copies/µg total RNA) was 62%, and the rate of the other 34 patients with low EphB4 (cases with ephrinB2 histoscore <190; EphB4 mRNA levels \( <1.9 \times 10^5 \) DNA copies/µg total RNA) was 82%. There was a significant difference \( (P < 0.01 \) in ephrinB2 and \( P < 0.05 \) in EphB4) between the 60-month survival rates of the 34 patients with high or low histoscores and mRNA levels of ephrinB2 and EphB4, as shown in Figure 3.

**Discussion**

As all Eph receptors have an extracellular portion (ectodomain), which contains the ligand-binding domain at the N terminus, and a cytoplasmic portion with the tyrosine kinase domain, it is obvious that when ephrinBs bind EphBs, the ephrinBs activate the EphB receptors, and the cells expressing ephrinB also demonstrate an increase in intracellular signaling that is due to the attachment of receptors and all known ephrin ligands.
Figure 2. (a) The histoscores and mRNA levels of ephrinB2 and EphB4 in normal endometria and uterine endometrial cancers classified according to clinical stages. Clinical stages of uterine endometrial cancer were assessed according to International Federation of Gynecology and Obstetrics (FIGO) classification. Each level is the mean ± standard deviation (SD) of nine determinations. Alive and deceased cases are numbered in ○ and ●, respectively. NE, normal endometrium; *P < 0.01, **P < 0.05. (b) The histoscores and mRNA levels of ephrinB2 and EphB4 in normal endometria and uterine endometrial cancers classified according to histological grades. Histological grades of uterine endometrial cancer were assessed according to FIGO classification. Each level is the mean ± SD of nine determinations. G1, well-differentiated adenocarcinoma; G2, moderately differentiated adenocarcinoma; G3, poorly differentiated adenocarcinoma. Alive and deceased cases are numbered in ○ and ●, respectively. NE, normal endometrium; *P < 0.01, **P < 0.05. (c) The histoscores and mRNA levels of ephrinB2 and EphB4 in normal endometria and uterine endometrial cancers classified according to depth of myometrial invasion. Depth of myometrial invasion of uterine endometrial cancer was assessed according to FIGO classification. Each level is the mean ± SD of nine determinations. A, tumor limited to the endometrium; B, invasion to less than half the myometrium; C, invasion to more than half the myometrium. Alive and deceased cases are numbered in ○ and ●, respectively. NE, normal endometrium; *P < 0.01, **P < 0.05.
EphB4 in tumor cells indicates that ephrinB/EphB signaling drives interactions among the tumor cells. Such interactions may result in destabilization, which can also affect cell–matrix attachment, and thereby promote invasion and metastasis [13]. Coexpression of ephrinB2 and EphB4 was found in the colon carcinoma [38] and cancer cell lines and tumors of small-cell lung carcinoma and neuroblastoma [39]. As a molecular mechanism of biological effect through autocrine stimulation and coexpression of c-kit and its ligand, the stem cell factor, was shown [40]. In the present study, coexpression of ephrinB2 and EphB4 significantly increased according to clinical stage, histological grade and myometrial invasion of the uterine endometrial cancer. The survival rate of the patients with higher coexpression of the ephrinB2 and EphB4 is significantly poor than that of the patients with lower coexpression. Our data indicate autocrine stimulation of the tumor advancement in uterine endometrial cancer mediated through second messengers, that are not figured out yet, of ephrinB2 and EphB4. In conclusion, ephrinB2 and EphB4 potentiate tumor progression with dedifferentiation and myometrial invasion, leading to poor prognoses. Therefore, ephrinB2 and EphB4 might work on tumor advancement and may be recognized as a novel prognostic indicator for uterine endometrial cancers. In addition, an attractive therapeutic strategy might be developed to block the ephrinB2/EphB4 signaling pathway using soluble ephrinB2 in the future.

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references


