Gynecologic Oncology 125 (2012) 427–432

Contents lists available at SciVerse ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno

Expression of 67-kDa laminin receptor was associated with tumor progression and poor prognosis in epithelial ovarian cancer

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ARTICLE INFO

Article history:
Received 11 October 2011
Accepted 18 January 2012
Available online 28 January 2012

Keywords:
67-kDa laminin receptor
Laminin
Molecular target
Prognosis
Ovarian cancer

ABSTRACT

Objective. 67-kDa laminin receptor (67LR) has been identified as a prognostic biomarker for a variety of human cancers. We investigated the clinical significance of 67LR expression and its functional role in epithelial ovarian cancer (EOC).

Methods. 67LR expression was evaluated by immunohistochemistry in 62 patients with EOC. We assessed the correlation of 67LR expression with clinical characteristics. *In vitro* experiment was performed for 67LR with inhibition using siRNA to evaluate its role in cell survival, apoptosis, and invasion in EOC cells.

Results. 67LR was predominantly expressed on the cell membrane in the majority of EOC samples (45/62, 73%). 67LR expression was significantly correlated with advanced stage ($P=0.001$). Patients with 67LR expression had shorter progression-free survival among all the patients ($P=0.010$) and in particular among patients with advanced stages ($P=0.046$). When 67LR expression was inhibited by siRNA in EOC cells (HeyA8 and A2780), there was a significant decrease of cell proliferation and invasion as well as increase of apoptosis.

Conclusion. These findings suggest that 67LR expression may play an important role in tumor progression into advanced stage with poor prognosis in EOC and down-regulation of 67LR on tumor cells may be a therapeutic target in those patients.

Introduction

Epithelial ovarian cancer (EOC) continues to be the leading cause of death among gynecologic malignancies because it is usually diagnosed in advanced-stage of the disease [1–4]. The transition from early to advanced-stage EOC is a critical determinant of survival. Therefore, investigation into the molecules that contribute to progression and metastasis of ovarian tumors is urgently needed.

Laminin, one of the major component of basement membranes, promotes important biological activities such as cell attachment, migration, proliferation, growth, and invasion [5]. Among the several cell surface proteins that are able to interact with laminin, 67-kDa laminin receptor (67LR) is involved in the chemotactic migration of malignant cells toward laminin [6]. Compared with the normal tissue counterpart, 67LR expression is increased in a variety of human cancers, including breast [7, 8], gastric [9, 10], colorectal [7, 11, 12], lung [13, 14], thyroid [15], pancreas [16], and prostate [17]. Furthermore, this increase in 67LR is associated with disease recurrence or survival even in some cancers [8–10, 16, 17]. There are only three studies which have focused on 67LR in EOC, the studies showed that 67LR expression is correlated with the presence of malignancy [18, 19], high histologic grade [20], advanced stage [19], and suboptimal debulking [18]. However, little is known about the prognostic significance of 67LR and its functional role in tumor progression of EOC. In the present study, we evaluated the association between prognosis and expression of 67LR in EOC with immunohistochemistry and its functional role on cell proliferation, apoptosis and invasion with siRNA in EOC cells.

Materials and methods

Tumor samples

This study was approved by the Institutional Review Board of Samsung Medical Center. We obtained formalin-fixed, paraffin-embedded tissues from the primary ovaries obtained during surgery from women with EOC (n = 62). As controls, we also obtained normal

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doi:10.1016/j.ygyno.2012.01.030

ovarian tissues (n = 4) from women who underwent hysterectomies for benign disease. All operations were performed in the Department of Obstetrics and Gynecology at Samsung Medical Center between October 2003 and November 2005. All women with cancer underwent primary maximal cytoreductive surgery followed by intravenous paclitaxel (175 mg/m^2) or docetaxel (75 mg/m^2) plus carboplatin (AUC 5) combination chemotherapy every 3 weeks for 6–8 cycles. Surgical staging was established according to the International Federation of Gynecology and Obstetrics (FIGO) system. Eleven tumors were classified as stage I, 6 were stage II, 42 were stage III, and 3 were stage IV. Debulking status was defined according to the size of the nodules left in the peritoneal cavity after surgery (<1 cm, 37 patients; ≥1 cm, 25 patients). Median follow-up time of the study cohort (n = 62) was 63.6 months (range, 2.6–108.3 months). At last follow-up, 36 (58%) relapsed with a median time of 21.0 months (range, 3.0–82.0 months).

Cell lines

Human EOC cell lines (HeyA8, SKOV3ip1, 2774, OVCAR3, PA-1, and A2780) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human embryonic kidney (HEK) 293 cells, which serves as a positive control cell line for 67LR [21], were obtained from the ATCC. All cell lines, with the exception of PA-1 and HEK 293 (MEM medium), were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 0.1% gentamicin sulfate (Gemini Bioproducts, Calabasas, CA, USA) in 5% CO_2 at 37 °C.

Immunohistochemistry

Immunohistochemical staining was performed with the standard peroxidase/DAB method (DakoCytomation, Glostrup, Denmark) on formalin-fixed, paraffin-embedded tissue sections. 67LR expression was detected by primary rabbit polyclonal 67LR antibody (NBP1-33002; Novus Biologicals, Littleton, CO, USA). Immunohistochemical procedures were performed as described previously [22]. The antigen–antibody reaction was detected using the Dako REAL™ Envision™ Detection system (peroxidase/DAB K5007; Dako, Glostrup, Denmark) according to the manufacturer's protocol. Tissue sections were lightly counterstained with hematoxylin, then examined by light microscopy. Anti-rabbit IgG (AI-2000, Vector Laboratories, Burlingame, CA, USA) was used in place of the primary antibody as a negative control. Two pathologists (SYS and COS) blindly reviewed slides and evaluated the immunohistochemical staining without knowledge of the clinical outcome by a pathologist. The scoring method described previously [12, 23, 24] was followed for the evaluation of 67LR expression. A case was considered positive when more than 1% of epithelial cells in 10 random, high-power fields were positively stained.

Western blot analysis

Cells were lysed in PRO-PRE Protein Extraction Solution (Intron Biotechnology, Seongnam, Korea). Protein lysates were separated in 12% acrylamide gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Hybond-ECL nitrocellulose filter paper (Amersham, Buckinghamshire, UK). Membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h at room temperature. Protein bands were probed with 67LR antibody (Novus Biologicals) and anti-tubulin antibody (Epitomics, Burlingame, CA, USA) and labeled with horseradish peroxidase-conjugated anti-rabbit antibody (Amersham, Piscataway, NJ, USA). Bands were visualized by enhanced chemoluminescence using an ECL kit (Amersham) according to the manufacturer's protocol.

Transfection of 67LR siRNA and proliferation assay

The validated form of 67LR siRNA 5′-GGAGGAAUUUCAGGGUGAA-3′ and AccuTarget™ negative control siRNA 5′-AAGUCGGACAAACAC CUCUAC-3′ were obtained from Bioneer (Daejeon, Korea). HeyA8 and A2780 cells were seeded at 2×10^3 cells/well in a 96-well microplate in RPMI 1640 with 10% FBS. All siRNAs were transfected at a final concentration of 20 nM using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Then cells were incubated at 37 °C for 48 h.

For the proliferation assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) solution (Amresco, Solon, OH, USA) was subsequently added to each well. After 4 h of additional incubation, the medium was discarded, 100 μl of acidic isopropanol (0.1 N HCl in absolute isopropanol) was added, and the plate was shaken gently. Absorbance was measured on an enzyme-linked immunosorbent assay (ELISA) reader at a test wavelength of 540 nm.

Apoptosis assay

For the apoptosis assay, we used an active caspase-3 ELISA assay (#KHO1091; Invitrogen). Cells were seeded in a 6-well plate (1×10^4 cells in 3 ml of media per well), and incubated overnight to allow the cells to attach to the plate. After 24, 48, and 72 h of siRNA transfection, the medium was removed by suction. The cells were lysed with lysis buffer. The apoptotic activity was determined for each well according to the manufacturer's protocol.

Invasion assay

Invasion was measured using a 24-well, 8-μm pore size Trans well chamber assay (Corning Inc., Corning, NY, USA) according to the manufacturer's protocol. Briefly, filters were pre-coated on the upper side with Matrigel (20 μg per well; BD Biosciences, San Jose, CA, USA). The cells (1×10^5) were seeded in serum-free RPMI 1640 in the upper compartment of the chambers for 16 h at 37 °C. After incubation, cells invading the bottom surface of the filter were fixed and stained with 0.1% crystal violet in 20% methanol. The invasiveness was determined by counting the penetrating cells under a microscope at ×200 magnification from 10 random fields per well.

Statistical analysis

The Pearson's chi-square and Fisher's exact tests were used to assess the statistical significance of the association between 67LR expression and clinicopathologic parameters. Kaplan–Meier curves were plotted to assess the effects of 67LR expression on progression-free survival. Survival curves were compared using the log-rank test. Cox proportional hazard models were used to assess the prognostic significance of 67LR expression and several clinicopathologic parameters. *P*-values less than 0.05 were considered to be statistically significant. All statistical analyses were performed using SPSS (version 15.0; SPSS, Inc., Chicago, IL, USA). Each *in vitro* experiment was carried out at least three times in triplicate.

Results

67LR expression and prognosis of EOC

We assessed 67LR expression using immunohistochemistry with 62 EOCs including 49 serous, 10 endometrioid, and 3 mucinous subtypes. Figure 1 shows representative results of immunohistochemical staining for 67LR. 67LR was predominantly expressed on the cell membrane, and occasionally within the cytoplasm as punctuated staining. Immunohistochemical analysis demonstrated that 67LR expression was not observed in all of the normal ovarian tissues