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ScienceDirect
Gynecologic Oncology 105 (2007) 563–570
Gynecologic Oncology
www.elsevier.com/locate/ygyno

Overexpression of folate binding protein and mesothelin are associated with uterine serous carcinoma☆

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Received 23 January 2006
Available online 2 April 2007

Abstract

Purpose. Folate receptor alpha (FOLR1) is a membrane bound receptor involved in the transport of folate as well as other regulatory cellular processes. The purpose of this study was to examine the expression of FOLR1 in uterine cancers and to identify changes in gene expression that are associated with overexpression of FOLR1.

Experimental design. Fifty-eight frozen uterine cancer specimens were stained for FOLR1 using immunohistochemistry and results were correlated with transcript expression noted on quantitative PCR. Total RNA from 16 cases of uterine serous carcinoma (USC) was analyzed for gene expression using the Affymetrix HG-U133A and HG-U133B GeneChip set. USCs overexpressing FOLR1 were compared to cancers with an absence of FOLR1 using binary comparison and template matching of data was used to identify genes that correlate with FOLR1 expression. Selected targets from this analysis were evaluated by quantitative PCR as well as in an independent set of USC represented in quadruplicate on a tissue microarray (TMA).

Results. Overexpression of FOLR1 was observed in 11/16 (69%) of USC and 0/10 normal endometrium cases using frozen tissue specimens. Binary comparison between FOLR1 positive and negative cases identified 121 genes altered by 2-fold at $p<0.01$ of which 45 are well correlated with FOLR1 expression pattern. Using quantitative PCR, both mesothelin (MSLN) and PTGS1 (COX1) were significantly increased in FOLR1 overexpressing tumors ($p=0.014$ and $p=0.006$ respectively). TMA confirmed that overexpression of FOLR1 and MSLN respectively occurred in 23/48 (48%) and 17/54 (32%) of pure USC.

Conclusion. Both FOLR1 and MSLN are cell surface targets that are co-expressed at high levels in USC and are appealing targets for biologic therapy.

Keywords: Folate binding protein; Papillary serous; Gynecologic tumors

Introduction

The folate receptor is a glycosol-phosphatidylinositol linked membrane protein that facilitates transfer of folate into the cell. Because folate provides the one-carbon units necessary for methylation of DNA, proteins, and phospholipids, cellular supply of folate can be important in the regulation of cellular processes [1]. Although a principal physiologic function of the folate receptor is to transport folate across the cellular membrane,

☆ Presented at the 36th Annual Meeting of the Society of Gynecologic Oncologists, Miami, 2005.

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0090-8258/$ - see front matter © 2007 Published by Elsevier Inc.
doi:10.1016/j.ygyno.2006.10.063

it also may play a role in cellular proliferation, independent of its role in folate uptake [2,3]. The association between FOLR1 overexpression and unregulated cellular proliferation has not been elucidated in any significant detail. Multiple studies have demonstrated increased FOLR1 expression in as many as 90% of ovarian cancers, particularly those of papillary serous histology [4–7]. However, there is limited information regarding the expression of FOLR1 in uterine cancer.

In a previous microarray study, we reported that the FOLR1 transcript was overexpressed in a significant proportion of endometrial adenocarcinomas [8]. Quantitative PCR indicated that the levels of FOLR1 transcript among uterine serous cancers (USC) were 60-fold greater than in normal endometrium. The purpose of the current investigation was to confirm whether overexpression of FOLR1, at both the transcript and protein level, occurs in USC and to determine which genes are typically co-expressed with FOLR1.

Materials and methods

Frozen tissue specimens

Fresh frozen tissue samples were obtained from 58 patients (16 uterine serous carcinomas, 13 endometrioid adenocarcinomas and 19 mixed mullerian tumors (MMMT) undergoing surgery for uterine cancer at Duke University Medical Center. Tissue specimens were evaluated by H&E to confirm that the specimen to be analyzed contained greater than 50% cancer cells. During preparation of the specimens for analysis, care was taken to macroscopically dissect the cancers away from any adjacent myometrium. In addition, specimens of normal endometrium were obtained from age-matched women undergoing hysterectomy for benign indications. Normal endometrium was examined and glandular epithelium and stoma was grossly dissected from 10 of the specimens. All tissues were obtained after receiving consent described in a tissue banking process approved by that Institutions' Review Board (IRB) who granted authorization to collect and study the tissue samples.

Immunohistochemistry for FOLR1 using frozen specimens

Tissue specimens were cut into 5 μm sections and mounted on untreated glass slides prior to fixation. After being re-hydrated and washed in buffers, the slides were incubated with the primary antibody (Mov 18/ZEL, Alexis Biochemicals) (diluted 1:1000) at room temperature for 3 h. Antigen retrieval was not performed. Following washing in buffers, the slides were incubated with 0.3% Hydrogen peroxide in PBS for 40 min to block endogenous peroxidase activity. The slides were incubated with biotinylated horse antimurine immunoglobulin (Vector, Burlingame, CA, USA) and then with Vectastatin ABC Mix (Vector, Burlingame, CA, USA) at room temperature for 30 min. Additional washing with buffers was performed prior to the slides being counterstained with Methyl Green. The slides then underwent a series of dehydration steps with 95% and 100% ethanol, and were then mounted with coverslips. Negative controls were used with each run and consisted of the ES-2 cell line, normal endometrium, and a PBS slide lacking MOV18 antibody. Positive controls included the SKOV-3 cell line, which had strong expression by Taqman analysis, and a set of 15 serous ovarian cancer specimens that were identified as being positive for FOLR1 during optimization of the immunohistochemical assays. The intensity of FOLR1 staining was graded from 0 to 3 and the proportion of tumor cells staining at each intensity level was also recorded. Cases in which at least 30% of the cells demonstrated a staining intensity of 2–3 were considered positive.

Microarray analysis

The global gene expression for each of the 16 frozen USCs was assessed using the Affymetrix HG-U133A and HG-U133B (45,000 gene transcripts covering 28473 UniGene clusters) [9]. The global gene expression of these cases has been previously reported as part of a larger set of uterine cancers undergoing oligonucleotide microarray analysis [9]. This database was utilized to determine FOLR1 transcript expression for the 16 cases of uterine cancer as well as facilitate binary comparison and template matching according to FOLR1 expression.

Binary class comparison was used to compare global gene expression from samples with positive folate binding protein staining versus those without immunohistochemical staining. The analysis was performed using BRB Array tools software (BRB Array tools ver. 3.0c, Richard Simon, Amy Peng, Biometric research branch, NCI, NIH, http://linus.nci.nih.gov/BRB-ArrayTools.html). Statistical calculations were performed on the logarithmic values of signals. Differentially expressed genes were identified by parametric Student's *t*-tests on genes having at least 5% or more present calls.

For Template Matching, 29272 genes having at least 1 present call in the serous samples were used for the analysis. Two templates were considered: one was idealized for FOLR1 positive and negative cases as +1 and −1 respectively; and the other was actual FOLR1 expression relative to normal endometria. The genes were selected if the correlation coefficient between either of the templates and gene expression pattern is >0.7 (or ≤ 0.7), *t*-test $p<0.01$, and the ratio is 2-fold for comparison between FOLR positive and negative cases. The expression data were displayed relative to average normal endometria expression. The cluster was color-coded using red for up-regulation from normal endometria and green for down-regulation.

Validation of gene expression

Quantitative PCR

The concentrations of select genes chosen for validation of gene expression were determined using the standard curve method for normalization and the results compared with average threshold PCR amplification cycle time (C_T) of normal endometrial samples. The relative gene expression (on logarithmic scale to base 2) of selected genes in each histologic group was compared to normal endometria. Gene expression assays for the analysis of samples were purchased from Applied Biosystems, Foster City, CA.

Tissue microarray

Paraffin tissue samples used for tissue microarray were collected from patients diagnosed with and treated for uterine malignancy between January 1st 1980 and July 31st 2003 at the Arthur James Cancer Hospital of the Ohio State University (OSU). The creation of the uterine cancer tissue microarray and use of this resource for the purposes of this study was performed following protocol approval by the OSU Institutional Review Board. An endometrial cancer TMA was constructed from the primary tumors harvested from the hysterectomy specimens of 485 patients. The specimens used for the tissue microarray represented various histologic types and stages of endometrial cancer. The presence of tumor tissue on the arrayed samples was verified on a hematoxylin–eosin-stained section and each case was represented in quadruplicate on the TMA. Specimens for controls consisted of 50 secretory endometrium, 50 proliferative endometrium, 50 normal cervix, and 50 normal ovaries.

Immunoperoxidase staining was performed on each formalin-fixed, paraffin-embedded TMA cut at 4 μm and placed on positively charged slides. Slides were placed in a 60 °C oven for 1 h, cooled, then deparaffinized and rehydrated using xylene and graded ethanol solutions to water. All slides were quenched for 5 min in a 3% hydrogen peroxide solution in methanol to block for endogenous peroxidase. Antigen retrieval was performed by a steamer heat method in which the specimens were placed in a citric acid solution (Target Retrieval Solution, pH 6.1; Dako Cytomation, Carpinteria, CA), for 30 min at 94 °C using a vegetable steamer.

Multiple attempts were made to use the variations of the MOV-18 assay (used for initial staining of frozen tissue specimens) to evaluate FOLR1 expression in the paraffin tissue specimens on TMA without success. This antibody was robust in frozen tissue but is suboptimal in the staining of paraffin fixed tissue. Subsequent staining of the endometrial cancer TMA using the Pu-17 antibody (Endocyte Inc.) was successful in the staining of paraffin fixed tissues for FOLR1 expression. The Pu-17 antibody was diluted 1:50 and incubated for 60 min. Immunohistochemical staining of MSLN was performed using (clone 5B2, Novocastra Laboratories) at a 1:10 dilution. All paraffin tissue slides underwent antigen retrieval in a vegetable steamer containing citrate buffer prior to being