Mutant Epidermal Growth Factor Receptor in Benign, Borderline, and Malignant Ovarian Tumors

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Abstract

Purpose: Dysfunction of the epidermal growth factor (EGF) complex is essential to the growth and development of many human tumors. Overexpression of the EGF receptor (EGFR) is a characteristic finding in a considerable number of solid tumors and often signalizes poor prognosis. There is a major disagreement among researchers about both the frequency and possible clinical importance of EGFR overexpression in ovarian cancer. The type III variant of EGFR (EGFRvIII) is a mutant with a deletion. Contrary to the wild-type, it is constitutively active. EGFRvIII has not been found in normal tissue, and consequently, it is an attractive tumor-specific candidate for molecular targeted treatment. The literature dealing with this mutation in ovarian cancer has been very sparse.

Experimental Design: Tissue from 225 patients who underwent surgery for a pelvic mass was collected consecutively. The samples included 99 ovarian/peritoneal/tuba cancers, 17 ovarian borderline tumors, 66 benign ovarian tumors, 15 other cancer types, 24 normal ovarian biopsies, and 4 miscellaneous. The presence of EGFRvIII was investigated both by PCR analyses for EGFR-vIII gene expression and with protein analysis by Western blots.

Results: None of the tissue samples was positive for the EGFRvIII mutation neither at the mRNA level nor at the protein level.

Conclusions: The EGFRvIII mutation seems to be very rare in ovarian tissue. Our data indicate that EGFRvIII is not a part of the malignant phenotype in ovarian cancer and should not be pursued as a therapeutic target for treatment of this disease.

The epidermal growth factor receptor (EGFR) system is essential to cell proliferation, migration, and adhesion. EGFR is commonly overexpressed in many human malignancies and is often associated with an aggressive phenotype implying a poor prognosis. Therefore, much attention has been paid to the receptor as target for antineoplastic therapy. A monoclonal antibody is now approved and commercially available for treatment use in daily clinic (1, 2).

The EGF complex is also of clinical interest in ovarian cancer. Overexpression of the EGFR occurs frequently in ovarian cancer (3–7) and has been associated with poor prognosis in several studies (5, 7); however, the results are diverging.

A mutant and constitutively active EGF receptor, also named EGFRvIII, which lacks a portion of the extracellular ligand-binding domain, has been detected in some tumors. This mutant contains a deletion of exons 2 to 7 (801 bp) from the extracellular domain, making it unable to bind EGF or other EGFR-binding ligands. Nevertheless, this deletion has an activating effect on the receptor, rendering it constitutively phosphorylated and active (8, 9). The EGFRvIII was first characterized in glioblastomas (10, 11) but has been detected in many other tumor types, including head and neck cancer, non–small cell lung cancer, and prostate cancer (12–17).

The literature describing its potential presence in ovarian cancer is rather sparse, and thus far, only one other study (13) has investigated the expression of EGFRvIII in ovarian cancer. The authors reported the presence of EGFRvIII in 24 of 32 (75%) ovarian cancer samples.

Another two in vitro studies have investigated EGFRvIII in ovarian cancer, both of which investigated the aggressive behavior of ovarian cancer cell lines transfected with an EGFRvIII construct (18, 19) and not the potential presence in human ovarian cancer.

Because EGFRvIII is found only in tumor cells and not in normal tissue (13, 16), it is an attractive candidate for targeting anticancer therapy against tumor cells without affecting cells in normal tissue (20, 21).

New biological agents in combination with traditional debulking surgery and chemotherapy are a novel approach in...
cancer treatment and it may result in further improvement in survival for patients with epithelial ovarian cancer.

The primary aim of the present study was to investigate the frequency of EGFRvIII mutation in ovarian cancer, borderline ovarian tumors, benign ovarian tumors, and normal ovarian tissue.

Materials and Methods

Patient data

The study included 225 patients undergoing surgery for a pelvic mass. Fresh-frozen tissue and tissue fixed in an immediate RNA stabilization reagent (RNAlater, Qiagen GmbH) were collected consecutively during the period from March 2005 to April 2007. Twenty-four patients undergoing oophorectomy and/or hysterectomy for benign nonovarian diseases served as controls. Histopathologic examination confirmed that their ovaries were normal.

Tissue samples were snap frozen in liquid nitrogen and stored at -80°C until investigation. Tissue in RNAlater was stored at -20°C as recommended by the manufacturer.

The Danish Biomedical Research Ethics Committee and the Danish Data Protection Agency approved the study. From each subject, oral and written informed consent was given to collect a tissue sample during their planned surgery. The consent was obtained before surgery.

EGFRvIII gene expression

RNA purification. For purification of RNA from the tissue fixed in RNAlater, the Maxwell 16 Total Purification kit (Promega) was used according to the manufacturer’s instructions. In brief, the tissue was cut into small pieces and 400 μL of RNA lysis buffer were added followed by homogenization using a FastPrep FP120 (Bio 101, Qbiogene) and cooled on ice. DNA dilution buffer (670 μL) was then added to precipitate sample debris followed by 75 μL clearing agent to remove genomic DNA. The sample was subsequently heated at 70°C for 3 min, mixed, incubated for 5 min at room temperature, transferred to the clearing column, and cleared by centrifugation. The lysate was transferred to well #1 of the Maxwell RNA cartridge.

RNA concentration was measured in 96-well format by Quant-IT Ribogreen RNA Quantitation kit (R-11490, Invitrogen, Molecular Probes) according to the manufacturer’s instructions.

Fluorescence was measured in triplicate (FL600 Microplate Fluorescence Reader, BioTek Instruments). From the standard curve, the fluorescent signal was converted to a concentration of RNA (ng/μL).

cDNA synthesis and quantification of EGFRvIII mRNA expression. cDNA synthesis was done using Primer “random” (Roche Applied Science) according to the manufacturer’s instructions [200 ng RNA to a final volume of 20 μL cDNA (10 ng/μL), buffer II, MgCl2, murine leukemia virus reverse transcriptase, and RNase inhibitor (Applied Biosystems)].

Expression of EGFRvIII mRNA was measured using real-time PCR on the ABI Prism HT 7900 Sequence Detection System and Taqman (Applied Biosystems).

The PCR mixture contained 2 μL cDNA, 12.5 μL Universal PCR master mix, 900 nmol/L of each primer, and 200 nmol/L Taqman probe in a final volume of 25 μL. The cycling conditions for PCR amplification were done according to the manufacturer’s instructions. All quantifications of mRNA were carried out in duplicate.

To detect the deleted region of EGFRvIII, primers designed to flank the deletions of exon 2 to 7 were used. These primers were designed using a Primer express program and purchased from Applied Biosystems. The forward and reverse primer sequence was 5’-GGCTCTGGAGAAAGAAGGTAATT-3’ and 5’-CGCTTCTCTCCTCATC-3’, respectively, and the probe sequence was 6-FAM-TGACAGATCACGGCTC-MGB.

Quantification was done by using a standard curve obtained from isolated RNA from the EGFRVIII-positive cell line NR6M. The EGFRVIII-positive cell line NR6M was a kind gift from Dr. Darrel Bignar (Duke University Medical Center, Durham, NC).

For each PCR, negative [samples without cDNA (H2O)] and positive (samples of known concentration) controls were done.

EGFRvIII SDS-PAGE and Western blot

Extraction of protein. Tissue samples ( ~ 60 mg) were homogenized in 600 μL lysis buffer [20 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA (pH 7.4), 10% glycerol, 1% Triton X-100, protease inhibitors] using a modified program for Maxwell 16 Instrument (Promega). After homogenization, the lysate was left to stand at 4°C for 30 min followed by centrifugation at 16,000 x g for 15 min at 4°C. The supernatants were recovered and the protein concentration of each sample was determined using the bicinchoninic acid protein assay (Pierce).

PAGE and Western blotting. One-dimensional SDS-PAGE was done in 7.5% uniform gels using a Tris-glycine buffer system (Bio-Rad). Equal amounts of protein (80 μg) from tissue were subjected to PAGE in the presence of 2% SDS and 40 mmol/L DTT. For positive control, 2 μg of EGFRvIII-positive control (NR6M cell line) lysate along with 2 μg negative control (A431 cell lysate [BD Biosciences]), which express only the EGFR wild-type (EGFRwt), were also subjected to each PAGE.

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n (%)</th>
</tr>
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<tbody>
<tr>
<td>Age (y)</td>
<td>n (%)</td>
</tr>
<tr>
<td>&lt;40</td>
<td>17 (7.6)</td>
</tr>
<tr>
<td>40-65</td>
<td>121 (53.8)</td>
</tr>
<tr>
<td>&gt;65</td>
<td>87 (38.7)</td>
</tr>
<tr>
<td>Histopathologic diagnosis</td>
<td>n (%)</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>99 (44.0)</td>
</tr>
<tr>
<td>Serous</td>
<td>70 (70.7)</td>
</tr>
<tr>
<td>Mucinous</td>
<td>3 (3.0)</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>8 (8.1)</td>
</tr>
<tr>
<td>Clear cell</td>
<td>4 (4.1)</td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>1 (1.0)</td>
</tr>
<tr>
<td>Mixed</td>
<td>1 (1.0)</td>
</tr>
<tr>
<td>Serous (tubal)</td>
<td>3 (3.0)</td>
</tr>
<tr>
<td>Serous (peritoneal)</td>
<td>5 (5.1)</td>
</tr>
<tr>
<td>Carcinosarcoma</td>
<td>3 (3.0)</td>
</tr>
<tr>
<td>Malignant Brenner</td>
<td>1 (1.0)</td>
</tr>
<tr>
<td>Borderline ovarian tumor</td>
<td>17 (7.6)</td>
</tr>
<tr>
<td>Serous</td>
<td>9 (52.9)</td>
</tr>
<tr>
<td>Mucinous</td>
<td>7 (41.2)</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>1 (5.9)</td>
</tr>
<tr>
<td>Benign ovarian tumor</td>
<td>66 (29.3)</td>
</tr>
<tr>
<td>Normal ovaries (controls)</td>
<td>24 (10.7)</td>
</tr>
<tr>
<td>Other cancers</td>
<td>15 (6.7)</td>
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<tr>
<td>Endometrial</td>
<td>4 (26.7)</td>
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<tr>
<td>Gastrointestinal</td>
<td>10 (66.7)</td>
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<td>Cervix</td>
<td>1 (6.7)</td>
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<tr>
<td>Miscellaneous</td>
<td>4 (1.8)</td>
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<tr>
<td>Acute and chronic tubal inflammation</td>
<td>2 (50.0)</td>
</tr>
<tr>
<td>Leiomyomatous tumor, uncertain malignant</td>
<td>1 (25.0)</td>
</tr>
<tr>
<td>Myometrial fibroma</td>
<td>1 (25.0)</td>
</tr>
<tr>
<td>FIGO</td>
<td>n (%)</td>
</tr>
<tr>
<td>I</td>
<td>17 (17.2)</td>
</tr>
<tr>
<td>II</td>
<td>10 (10.1)</td>
</tr>
<tr>
<td>III</td>
<td>60 (60.6)</td>
</tr>
<tr>
<td>IV</td>
<td>12 (12.2)</td>
</tr>
<tr>
<td>Histologic tumor grade</td>
<td>n (%)</td>
</tr>
<tr>
<td>Well differentiated (grade 1)</td>
<td>15 (16.1)</td>
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<tr>
<td>Moderately differentiated (grade 2)</td>
<td>31 (33.3)</td>
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<tr>
<td>Poorly differentiated (grade 3)</td>
<td>47 (50.5)</td>
</tr>
<tr>
<td>(NA/unknown: 6)</td>
<td></td>
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</table>
For subsequent analysis by Western blotting, proteins were electrophoretically transferred for 30 min at 100 V in 25 mmol/L Tris-amine, 192 mmol/L glycine (pH 8.3), 20% ethanol, and 0.01% SDS to a polyvinylidene difluoride membrane. The membranes were blocked overnight at 4°C with 20 mmol/L Tris-HCl, 137 mmol/L NaCl, and 0.1% Tween 20 (pH 7.6) containing 5% (w/v) skimmed milk.

EGFRwt and EGFRvIII were subsequently detected using a monoclonal mouse anti-human EGFR antibody, which recognizes both EGFRwt and EGFRvIII (concentration, 1 μg/mL; clone 31G7; Zymed), and horseradish peroxidase–conjugated goat anti-mouse IgG (DakoCytomation). Immunoreactive bands were detected with Immun-Star horseradish peroxidase chemiluminescent detection kit (Bio-Rad) followed by autoradiography.

For further validation, we did additional blots where EGFRwt and EGFRvIII were detected in two ways by two different antibodies on separate Western blots. First, we used the previously used monoclonal mouse anti-human EGFR antibody, specific for both EGFRwt and EGFRvIII (concentration, 1 μg/mL; clone 31G7), and horseradish peroxidase–conjugated goat anti-mouse IgG.

On the other Western blots, we used a highly specific monoclonal antibody against EGFRvIII, L8A4, at a concentration of 20 μg/mL. The L8A4 antibody was a kind gift from Dr. Darrel Bigner. For these validations studies, we used NR6M as the EGFRvIII-positive control with the NR6W and NR6 cell lines as EGFRvIII-negative controls. NR6W is a cell line transfected with wild-type EGFR, so this cell line is a negative control for EGFRvIII but a positive control for EGFRwt. NR6 is an untransfected cell line that does not express EGFRwt or EGFRvIII.

Statistical analyses. Statistical analyses were done with the NCSS software (version 2001).7

Results

The patient characteristics are summarized in Table 1. The median age of the patients was 60.2 years (range, 20-88 years). Table 1 shows that the majority of the included cancer patients were in an advanced FIGO stage at the time of surgery. The most common histopathologic type was the serous tumor type.

EGFRvIII gene expression. From the 225 patients included in this study, 222 patients were available for EGFRvIII mRNA analysis (3 samples were missing). Another four samples were

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found to have a total RNA concentration of <3 ng/µL and were not used for further evaluation.

None of the remaining 218 samples was positive for the EGFRvIII mutation.

Figure 1 presents the standard curve made from dilutions of the positive NR6M cell line and also an amplification plot with representative ovarian samples.

**EGFRvIII SDS-PAGE and Western blot.** The EGFRvIII mutation was not present in any of the 225 analyzed samples. Neither malignant, borderline, benign, nor normal ovarian tissue samples showed positive bands for the EGFRvIII mutation by Western blotting. The Western blots with the EGFR antibody (clone 31G7), specific for both EGFRwt and EGFRvIII, detected both the positive control NR6M, the negative control NR6W, and the band for normal EGFR in the ovarian samples. The EGFRwt band for the NR6W cell line and for the tissue samples was detected at the expected size of 170 kDa. The antibody did not detect the complete negative control, NR6, which does not contain any EGFR (Fig. 2A).

The other Western blots where the highly EGFRvIII-specific antibody L8A4 was used for detection only detected the 145-kDa band in the positive NR6M control and did not detect any other EGFRvIII bands at 145 kDa nor in the negative controls or in any of the ovarian samples.

We did a dilution with decreasing amounts of protein of the EGFRvIII-transfected NR6M cell line for Western blot analysis to ensure that the results were not due to a low sensitivity. The Western blot detected EGFRvIII protein levels in the cell line down to a sensitivity of 0.25 µg protein, which corresponded to ~300 cells (Fig. 3).

We also tested the sensitivity with the ECL Advance (GE Healthcare Bio-Sciences Corp.), which is a Western blotting detection system designed to provide greater sensitivity in experiments involving a low concentration of the protein of interest. By using the ECL Advance system, our protein detection limit was 0.016 µg protein corresponding to ~19 cells (data not shown).

We used the ECL Advance system to detect EGFR in those samples that did not show detection of EGFR with the Immun-Star horseradish peroxidase chemiluminescent detection kit.

**Discussion**

Treatment of ovarian cancer is met with several difficulties, and as in many other tumor types, drug resistance is a major problem. Women with advanced ovarian cancer have a high risk of relapse after postoperative standard combination chemotherapy with carboplatin and paclitaxel. Patients with recurrent ovarian cancer are treated with palliative intent, and the clinical benefit based on current therapies in the relapsing setting is only modest.

New modalities with different mechanisms of action are urgently needed to improve the outcome of the disease.

Combination therapy with new molecular therapeutics targeting cell surface receptors may prove successful. Especially those targeting cell surface receptors primarily expressed by cancer cells and not by normal cells could potentially be a most useful approach. Studies investigating the EGFRvIII mutation suggest that this mutation is present in a variety of tumor types but not in normal tissue, which would make this mutant receptor an attractive target for anticancer therapy.
We did not find the presence of EGFRvIII in normal ovarian tissue and benign or borderline ovarian tumors. Surprisingly, the same applied to ovarian cancer. To validate the methods, we investigated the possible presence of the receptor by approaching both the protein and gene expression levels by Western blot and real-time PCR, respectively. The findings from the two different methods were consistently in agreement because neither method detected the mutated receptor in our material.

In addition, we tested for the presence of EGFRvIII protein by testing our samples with two different antibodies, but neither one detected any EGFRvIII in the samples.

Furthermore, our laboratory has previously validated the methods for detecting EGFRvIII in colorectal cancer by analysis of the same samples in another laboratory. The results were identical (22).

To ensure that our findings were not a result of too low sensitivity, we did a dilution of the EGFRvIII-transfected NR6M cell line for Western blot analysis and found a detection limit of 0.25 μg protein with the with Immun-Star horseradish peroxidase chemiluminescent detection kit and a detection limit indicated that the method can be expected to work.

Because neither method detected the mutated receptor in our samples, it cannot be excluded that the authors detected degraded protein and not EGFRvIII; alternatively, they may have used an antibody not specific for EGFRvIII.

Our study consisted of a rather large patient group with the presence of both normal, benign, borderline, and malignant ovarian tissue with well-defined clinical data and all with the histopathologic diagnosis confirmed by a skilled gynecologic pathologist, making this material unique and not likely a result of a small sample size.

In conclusion, our data indicate that the mutant receptor is not present in ovarian tissue. The EGFRvIII is not likely to be involved in the pathogenesis in ovarian cancer. The absence excludes its potential role as an attractive candidate for biological targeted anticancer therapy in ovarian cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank laboratory technologists Loni Frischknect, Birgit Lindholm Jensen, and Sara Stidsen and molecular biologist Mette Kusk Bøving for their skilled work with the gene expression and Western blot analysis; Dr. Darrel Bigner for the kind gift of the NR6, NR6W, and NR6M cell lines, along with the L8A4 EGFRvIII antibody; our collaborators at Aalborg University Hospital and the Pelvic Mass Group at Rigshospitalet, University of Copenhagen, for their kind contribution with patient material; and Irene Visinin (Department of Obstetrics and Gynecology, Reproductive Immunology Unit, Yale University, School of Medicine) for proofreading.

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