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ORIGINAL ARTICLE

Radioligand binding assay determination of epidermal growth factor receptor in ovarian tumours

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Summary

Purpose: *To introduce a quantitative method for determination of epidermal growth factor receptor (EGFR) expression in tissue samples taken from normal ovaries, benign and malignant ovarian tumours, convenient for routine tests.*

Materials and methods: *About 1g of tissue was taken intraoperatively from 136 patients; 105 of them had histologically verified ovarian tumours (64 malignant, 42 benign) and 30 had normal ovaries. The tissue was frozen, preserved and transported in liquid nitrogen (–196° C). The level and frequency of EGFR expression were determined by radioligand method, utilizing ^{125}I-labeled epidermal growth factor (EGF) and recombinant human EGF. The results were obtained as fmol bound EGF per mg protein from the membrane fraction. All samples having expression ≥3 fmol/mg were considered as positive.*

Results: *The frequency of EGFR expression was 52% (70/136 patients), with a mean level of expression 45 ±11 fmol/mg (range 0-1332). From the EGFR-positive patients with malignant ovarian tumours 21 (62%) had progressive disease (PD) while only 4 (13%) patients with negative EGFR had PD (p=0.001). The mean progression-free interval in the first group was 4 months, and in the second group it was 11 months (p=0.0028).*

Conclusion: *The proposed quanitative radioligand binding assay is easy to perform, rapid and well reproducible, and we recommend it for routine clinical use.*

Key words: EGFR, ovarian tumours, radioligand binding assay

Introduction

Cell survival is the result of a balance between cellular proliferation and programmed cell death (apoptosis). Cell membrane receptors and their associated signal transducing proteins control these processes. Of the numerous receptors and signalling proteins described, protein kinases modulate most signalling pathways. Along the cell surface about 60 receptors with intrinsic tyrosine kinase activity are described. First recognized in 1980, these receptors can be subdivided into several families: the family of EGFRs, fibroblast growth factor receptors, platelet-derived growth factor receptors, etc [1]. EGFR family consists of 4 members: EGFR (**H**uman **E**pidermal growth factor **R**eceptor) HER1, HER2, HER3 and HER4 [2]. Mature human EGFR (HER1) is a glycoprotein, consisting of 1186 aminoacids with molecular weight 170 kD, being a product of EGFR protooncogene [2]. In EGFR structure identified are an extracellular region (4 domains, 621 aminoacids), a transmembrane domain (23 hydrophobic aminoacids) and a cytoplasmic region, containing juxtamembrane tyrosine kinase and carboxyterminal domain. The ex-

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tracellular region's third domain is the EGF-binding domain [2,3].

EGFR is expressed in all epithelial and in many mesenchymal normal cells. It has a wide range of functions, depending on the tissue origin and state of differentiation [1]. The receptor exists as inactive monomer which, after ligand binding, is activated and forms homo- or heterodimers. The receptor's tyrosine kinase is activated, causing transphosphorylation of intracellular domains and initiating a cascade of intracellular events. Ras and mitogen-activated protein kinase are activated (mitogen-activated protein kinase – MAP-kinase). MAP-kinase transfers the signal from the cytoplasm to the nucleus. When it penetrates into the nucleus, cyclin D is accumulated, which together with cyclin-dependent kinases causes the cell transition from G1 to S-phase and initiates the cell division process [4-6].

EGFR is expressed in a number of malignant tumours, and is related to the disease stage, progression and survival [7-9]. The enhanced receptor activity caused by its overexpression, decreased degradation or mutation, is a major point in tumour pathogenesis [1,10].

EGFR significance for oncological practice is determined by the role of the receptor as a prognostic factor in a number of malignancies and its investigation as a target molecule for different therapeutic agents. The necessity of precise EGFR expression determination is seen in both cases. Different methods are described in the literature: biochemical, immunohisto-/immunocyto-chemical, flow cytometric, molecular-chemical [11-17]. Although immunohistochemical methods allow EGFR determination in very small samples, they only give qualitative, and in the best case, semi-quantitative results [18]. Among biochemical methods, both immunometric assays and assays involving saturation with labeled ligand (radioligand binding assay) are available. Unlike the immunometric assays, which can identify both inactive receptors and inactive fragments, the saturation assays allow a precise determination of only the biologically active receptors [18,19].

The objective of our study was to introduce a quantitative method for the determination of EGFR expression in tissue samples taken from normal ovaries, benign and malignant ovarian tumours, convenient for routine tests.

Patients and methods

Patients and tissue samples

Approximately 1g of tissue was taken intraoperatively from 136 patients; 105 of them had histologically verified ovarian tumours (64 malignant, 42 benign) and 30 had normal ovaries. The tissue was frozen, preserved and transported into liquid nitrogen (–196° C).

Chemicals

The following chemicals were used: 3-(^{125}I) iodotyrosyl recombinant human EGF with specific activity >750 Ci/mmol (Amersham Biosciences), and purified recombinant human EGF, purity >97% (R&D Systems).

All other materials were from Serva and Merck.

Radioligand binding assay

Tissue was cryohomogenized with liquid nitrogen in a porcelain mortar until fine powder was obtained (maximal mechanical cell destruction). Then, 1.5 ml 0.05 M phosphate buffer, pH=7.4, containing 0.066 EDTA, 0.001 monothioglycerol, and 10% glycerol were added. The homogenate was mixed well and centrifuged at 1,000 G for 10 min at 4° C. The supernatant was poured off as pellet, was resuspended in 1 ml from the same buffer and centrifuged again at 1000 G for 10 min at 4° C. The collected supernatants (cytosol), containing membrane fractions over which the receptor is situated, were centrifuged for 1 h at 20,000 G at 4° C. The supernatant was poured off as pellet and was resuspended in 25 ml of 0.25 mM Tris-HCl buffer, pH=7.4.

Samples were set up in pairs and each one of them contained 4.27 pmol of ^{125}I-labeled EGF. Samples in pairs were set up in parallel, containing 200 times more unlabeled EGF. Thus pairs of samples and competitions were formed.

Samples were incubated for 16 h at 26° C. They were supplemented with 2 ml 0.25 mM Tris-HCl buffer, pH=7.4, containing 1% human serum albumin and centrifuged for 15 min at 20,000 G at 4° C to separate bound from free labeled EGF. The supernatant fraction was poured off and the activity in the sediment was counted with automatic γ–counter for one min (LKB Co, Sweden).

The obtained mean radioactivity in the competitions was deducted from the obtained mean radioactivity in the samples. The impulses were transformed into quantity and were divided into the protein content. The results were obtained as fmol bound EGF per mg protein from the membrane fraction.

The protein content was determined by the bromphenol blue method with bovin serum albumin as standard [20].

All samples having expression ≥3 fmol/mg pro-

tein were considered as positive, and those with <3 fmol/mg protein as negative.

Experiments were conducted with 3 methods with varying quantity of cytosol, labeled and unlabeled EGF. Table 1 shows the differences between them. All other parameters were identical for the 3 methods. We obtained the best quantitative indices with method 3, thus we chose to apply it for our group of 136 patients.

EGFR as a quantitative and qualitative index was compared with the age of the patients, their distribution into age groups, the tissue morphological type, and the disease progression and progression-free survival for malignant tumours.

Statistical analysis

The quantitative variables were described by their mean value and standard error, and the qualitative ones by the respective shares of their different categories. The availability of relations and dependence between the signs was verified with ANOVA and x^2-test (Pearson). Data were processed by the SPSS software program for Windows v. 7.0.

Table 2. Patient clinical characteristics in relation to EGFR

Characteristic	*n*	*%*	*positive EGFR*
Age, years			
median (range)	54 (22-85)	–	–
Normal ovaries	30	22	13
Benign tumor	42	31	23
Malignant tumor	64	47	34
epithelial ovarian Ca	60	93.75	32
granulosa cell tumor	1	1.56	1
metastatic tumor	2	3.13	1
borderline malignancy	1	1.56	0
Stage of malignant disease			
I	9	14.0	4
II	6	9.4	2
III	34	53.2	16
IV	15	23.4	12
Clinical status			
CR[1]	18	28.1	1
PR[2]	10	15.6	5
SD[3]	5	7.8	3
PD[4]	31	48.5	25

[1]Complete response, [2]partial response, [3]stable disease, [4]progressive disease

Results

The study was carried out for a period of 3 years (January 2001 - December 2003). The median age of the studied group of 136 patients was 54 years (range 22-85); 64 (47%) patients had malignant ovarian tumours (36 serous, 12 mucinous, 7 endometrioid, 2 clear cell, and 3 mixed adenocarcinomas; 1 granulosa cell; 2 metastatic; and 1 borderline malignancy), 42 (31%) benign ovarian tumours (20 serous, 8 mucinous, 3 endometrioid adenomas; 6 thecoma-fibroma; and 5 mature dermoid cysts), and 30 (22%) morphologically normal ovaries. The 50-59 age group was predominant (44; 32% patients). Table 2 shows the patients clinical characteristics in relation to EGFR.

The frequency of positive EGFR expression in the whole group (136 patients) was 52% (70 patients). In the group with malignant and benign ovarian tumours, EGFR-positive were 34 (53%) and 23 (55%) respectively, and in the group with normal ovaries 13 (43%) patients (p=0.6).

In the group of 70 patients with positive EGFR expression 13 (18%) had normal ovaries, and 23 (33%) and 34 (49%) benign and malignant ovarian tumours, respectively (p=0.6, Table 3).

The mean value of EGFR expression in the analyzed group of 136 patients was 45±11 fmol/mg (range 0-1332). The mean level of receptor expression in patients with ovarian tumours was 45±14 fmol/mg (benign tumours 47±14 fmol/mg, malignant tumours 43±21 fmol/mg) and in those with normal ovaries it was 44±12 fmol/mg (p=0.1).

Table 1. Quantitative features of cytosol, labeled and unlabeled EGF for methods 1, 2 and 3

	Method 1		*Method 2*		*Method 3*	
	Sample	*Competition*	*Sample*	*Competition*	*Sample*	*Competition*
Cytosol (ml)	0.1	0.1	0.5	0.5	0.5	0.5
^{125}I-EGF (ml)	0.05	0.05	0.05	0.05	0.2	0.2
Buffer (ml)	0.05		0.05		0.2	
Unlabeled EGF (ml) – 200 times higher quantity		0.05		0.05		0.2

Table 3. EGFR positive expression in 70 patients

	n	*%*	*p-value*
Normal ovaries	13	18	
Benign tumor	23	33	0.6
Malignant tumor	34	49	

From the patients with malignant ovarian tumours having positive EGFR 21 (62%) had PD, while only 4 (13%) patients with negative EGFR had PD (p=0.001). The mean progression-free interval in the first group was 4 months, and in the second group it was 11 months (p=0.0028).

Discussion

The frequency of EGFR expression in ovarian tumour tissue varies greatly-from 13 to 82% [15, 21]. One of the main reasons of these variations is the usage of different methods for receptor testing. Henzen-Logmans et al. studied the EGFR expression in ovarian tumours by comparing radioligand binding assay with immunohistochemical methods using two monoclonal antibodies (MoAb)-2E9 and EGFR1. Both MoAbs gave positive results for the receptor in 77% of the tumours, while the radioligand binding assay was positive in 66%. The correlation between the maximum binding capacities of EGFR obtained from Scatchard plots and the percentage of positive tumour cells determined by the EGFR1 MoAb was statistically significant ($p < 0.0001$) but for the radioligand binding assay and 2E9 MoAb it was not ($p < 0.1$). The authors concluded that clinical studies are necessary to determine the possible prognostic impact of EGFR determined with either method, or whether a combination of both would give a better discrimination between high- and low-risk patients [22].

The radioligand binding assay and immunohistochemical method estimate different aspects of EGFR expression. The immunohistochemical method allows localizing of EGFR-positive cells, i.e. the receptor epitope, but the radioligand binding assay permits its quantitative measurement [14]. In our study we used the radioligand binding assay. The cut-off point defining EGFR expression as positive is 3 fmol/mg membrane protein. This is the lowest concentration that could be measured reliably [23].

We did not find statistically significant difference in the frequency of EGFR expression in patients with normal ovaries, benign and malignant ovarian tumours (p=0.6). In the group with epithelial tumours, EGFR-positive were 50 (54%) patients, as our results coincide with those obtained by Henzen-Logmans et al. (immunohistochemical analysis) [24].

We determined that patients with malignant ovarian tumours expressing EGFR progress significantly more frequently (p=0.001) and have statistically significant shorter mean progression-free survival (p=0.0028) compared to those who do not express it. In a series of 117 primary epithelial ovarian cancer studied by the radioreceptor assay, Scambia et al. also proved a significant correlation between EGFR-positive tumours and progression-free interval (p=0.0033) [25].

Different methods (radioligand binding assay, ELISA, flow cytometric method) are used for quantitative EGFR estimation. By ELISA and flow cytometry the results are presented as a number of receptor molecules/μg protein and number of EGFR binding sites per cell, respectively [11,26]. In radioligand binding assay the quantitative EGFR estimation is done in fmol bound EGF per mg protein from the membrane fraction.

The individual radioligand binding methods show differences in the ways for obtaining the membrane cell fraction, the time and temperature for incubation, as well as in the methods for separating the free from the receptor bound ^{125}I-EGF. Formento et al. centrifuged the tumour homogenate only once for 10 min at 800 G (2° C), then they centrifuged the supernatant (1 h, 105,000 G, 2° C) for separating the membrane fraction in the pellet [12]. A great part (76%) from the total content of EGFR in the cell goes in the nuclear fraction. The nuclear washing recovers about 53% of the cell membranes disrupted through homogenization as the subsequent two-fold centrifugation at 800 G for 10 min achieves almost their complete recovery [18].

We centrifuged the tumour homogenate for 10 min at 1,000 G (4° C), resuspended the pellet containing the nuclear fraction and centrifuged it again. After that, a satisfactory recovery of cell membranes in tumour cytosol was achieved.

The incubation time used by different authors varies from 1 to 45 h, and the temperature from 4 to 37° C [12,17,18,23]. Dittadi et al. studied 3 different incubation temperatures (4°, 26° and 37° C) and they found that the maximal specific binding of ^{125}I-EGF was at 26° C regardless of the incubation time (2-20 h). Furthermore, they also estimated the specific binding of ^{125}I-EGF with untreated placental membrane fraction at 26° C. The highest EGFR concentration values were obtained between 15th and 20th h, and they did not change significantly by 45th h [18]. Based on these

data, in our study we incubated the samples for 16 h at 26° C.

For separation of free from receptor bound ^{125}I-EGF, different methods are used (centrifugation, filtration, immunoprecipitation with MoAbs) [12,17-19,23]. Centrifugation is the method of choice when a great number of tissue samples should be tested [18]. The conditions allowing complete recovery of the receptor bound ^{125}I-EGF (100,000 G for 1 h) are not convenient for routine practice [19]. With low centrifugation speeds (800 G), a preliminary incubation with suspension of hydroxyapatite (1 h at room temperature) is used [23]. Centrifugation at 20,000 G for 30 min allows restoration of about 66% of the specifically bound ^{125}I-EGF in placental membranes [18].

For separation of bound from free-labeled EGF we added to the samples 2 ml 0.25 mM Tris-HCl buffer, pH=7.4, containing 1% human serum albumin and centrifuged them for 15 min at 20,000 G at 4° C.

The mean value of EGFR expression in our study was 45±11 fmol/mg. The differences between patients with normal ovaries, benign and malignant ovarian tumours were non significant (p=0.1). The data for malignant ovarian tumours (43±21 fmol/mg) are higher than those reported by Nagai et al. (5.8±11.1 fmol/mg) [27], which could be attributed to some aspects in the tumour homogenate processing (two-fold centrifugation for 10 min at 1,000 G) and separating of free from bound-labeled EGF by centrifugation for 15 min at 20,000 G at 4° C.

The study of EGFR as prognostic factor and also as a target structure for therapeutic agents is a precondition for working out different methodological approaches. The proposed by us quantitative radioligand binding assay is easy to perform, rapid and well reproducible, and we recommend it for routine clinical practice.

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