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Epidermal growth factor receptor gene expression and binding capacity in renal cell carcinoma, in relation to tumor stage, grade and DNA ploidy

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Abstract Epidermal growth factor receptor (EGFr) was studied in 19 renal cell carcinomas using competitive binding analysis and solution hybridization assay. EGFr binding capacity and EGFr mRNA expression were significantly higher in tumors in comparison with kidney cortex tissues. The EGFr binding capacity was higher in diploid than in aneuploid tumors. No differences in binding capacities or mRNA expression between different tumor grades or stages were demonstrated. It was concluded that EGFr is overexpressed in renal cell carcinoma, although with no relationship to tumor characteristics.

Key words EGF receptor · Renal cell carcinoma · mRNA binding capacity · DNA content

Various growth factors and growth factor receptors have been implicated in the development of a wide variety of human cancers [1]. Specifically, epidermal growth factor (EGF) has been suggested as a mitogenic hormone which may be involved in the regulation of proliferation and differentiation of normal and neoplastic cells. The epidermal growth factor receptor (EGFr) is a transmembrane glycoprotein and binding of EGF or transforming growth factor alpha (TGF-α) to this receptor results in an intracellular response leading to mitosis by activation of the tyrosine kinase [5]. The amount of EGFr appears to vary according to the cell type and stage of differentiation of many malignant tumors. For example, poorly differentiated bladder and breast tumors contain higher levels of EGFr than other histological grades of these cancers [16, 21]. In other tumors, for example prostatic cancer, it has been reported that a depletion in the number of EGFr binding sites correlates with the loss of differentiation of the tumor [19]. In renal cell carcinoma an overexpression of EGFr mRNA has been shown, which may be associated with the malignant transformation [15, 24]. However, in renal cell carcinoma, the impact on tumor biology of EGFr overexpression has not yet been evaluated.

The present investigation was performed in order to elucidate whether the expression of EGFr in renal cell carcinoma is related to other tumor parameters such as tumor grade, stage, DNA ploidy and disease progression.

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Materials and methods

Patients

This study was performed on surgical tumor samples from 19 patients with renal cell carcinoma operated upon with perifascial nephrectomy at the Department of Urology, University Hospital, Umeå, Sweden during 1990-1991. There were nine women and ten men. Their mean age was 57 years (range, 25-82 years). All patients were clinically staged according to UICC 1987 [14], using the histopathological spread and including chest radiography, ultrasonography and computed tomography of the abdomen, and bone scans in some patients. In six patients the tumor was confined to the kidney (T1-2,N0,M0), in six there was local spread (T1-3,N0-1,M0) and seven patients had advanced disease (T4 or M1).

Tissue samples

The tumor and kidney cortex tissues were obtained by surgical biopsies from the nephrectomized kidney with tumor. Tumor and kidney cortex samples were divided into two pieces, one for EGFr examination and the other part for histopathological evaluation. The tumors were classified in a four grade scale according to Skinner et al. [26].

DNA analysis

The method for flow cytometric DNA analysis has been described previously [18]. Briefly, the fresh samples were minced and stained using a propidium iodide solution. After staining the samples were filtered and run in a FACScan (Becton-Dickinson, Sunnyvale, Calif., USA). The kidney cortex tissue samples were used as standards for

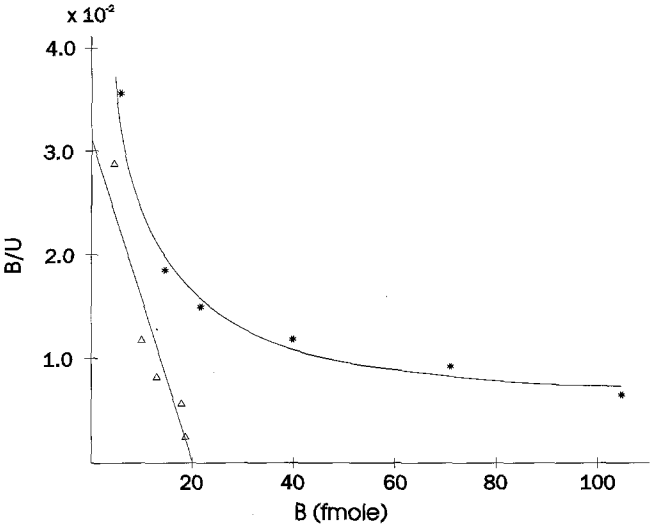

Fig. 1 Binding curve and Scatchard plot of specific hEGF binding capacity to cell membranes of one renal cell carcinoma (tumor 6/90). $K_d = 3.20 \times 10^{-9}$M; R = 19.9 fmol

diploidy. The tumor was denominated diploid (DNA index, 1.0) when only one peak was detected and aneuploid when two separate peaks were found, since it was assumed that all tumor samples contained normal as well as tumor cells. The FACscan had a coefficient of variation (CV) of < 3% for normal lymphocytes.

EGFr binding assay

Approximately 1 g of each tumor was homogenized with an Ultraturrax (TP 18/10) in 6 ml ice-cold 0.25 M sucrose using four 10-s periods allowing 30-s cooling intervals between bursts. After subsequent centrifugation for 15 min. at 1000 *g*, the supernatant was saved and the pellet was resuspended in sucrose, rehomogenized and centrifuged as above. The two supernatants were then combined and centrifuged for 30 min at 17000 *g*. The new supernatant was made up to final concentrations of 0.1 mol/l NaCl and 1 mmol/l $MgSO_4$ and centrifuged at 105000 *g* for 1 h in a Beckman ultracentrifuge. The pellet was resuspended in 4 ml 0.05 mol/l TRIS-HCl buffer (pH 7.4), mechanically homogenized and again centrifuged at 105000 *g* for 1 h. The new pellet was suspended in TRIS-HCl, 1 ml/g tissue weight, and frozen at −70°C until binding analysis.

One-hundred microliter aliquots of the membrane preparation were incubated for 16 h at +4°C with increasing concentrations of human EGF (0–100 ng) and a constant amount of ^{125}I-human-EGF (approx. 6000 cpm, specific activity, > 900 Ci/mmol, Amersham) in a final volume of 200 µl. After the incubation, a method using hydroxylapatite as described by Benraad and Foekens [2] was used to separate free and receptor-bound ligand. EGFr binding capacity was calculated according to the method of Scatchard after correction for the nonspecific binding [25].

Synthesis of EGFr probe and mRNA standard

The RNA probe used for solution hybridization and RNase protection assay was synthesized as described by Ekberg et al. [7]. The probe, a gift from Dr. J. Schlessinger (Israel), was a single-stranded antisense RNA probe synthesized by in vitro transcription of a 768 bp human EGFr cDNA (nct, 2318–3085), according to the sequence published by Ullrich et al. [28]. Labeling of the RNA strand was performed in vitro by incorporation of ^{35}S-CTP (Amersham, Buckinghamshire, England) in a reaction catalyzed by T7 polymerase (Promega, Wis., USA) [20]. DNA template was removed by DNase I treatment (Boehringer, Germany), and then the RNA probe was purified with phenol/chloroform extraction and ethanol precipitation.

An unlabeled sense RNA standard was synthesized in a similar way to the antisense RNA probe, using T7 polymerase transcription of the identical 768 bp fragment inserted in the opposite direction in the plasmid. In this transcription reaction using about 5 µg of the linearized DNA template a yield of 25–75 µg sense RNA was obtained (Promega Methodology Manual, Madison, Wis., USA) [20]. The RNA pellet obtained after DNaseI treatment was dissolved in 50 µl diethylpyrocarbonate-treated water. The sense RNA concentration of the sample was measured on a 100-fold dilution using a UV light spectrophotometer at 260 and 280 nm wavelength. Typically values of 0.1–0.3 were obtained at 260 nm, and 260-nm/280-nm values were close to 2.0.

Preparation of total nucleic acid (TNA)

Tissues were homogenized with a Polytron Kinematica, Luzern, Switzerland (3 × 10 s at medium setting) in a SET buffer containing 1% (SDS) sodium dodecyl sulfate, 10 mM ethylenediaminetetraacetic acid EDTA and 20 mM TRIS-HCl (pH 7.5). TNA was then prepared by digestion of the homogenized tissue with proteinase K and subsequent extraction with phenol/chloroform [6]. The nucleic acid was then precipitated with ethanol and dissolved in SET buffer. The amount of DNA was determined by fluorometry.

Solution hybridization assay

To quantify EGFr mRNA, the ^{35}S-labeled human antisense RNA probe was hybridized to TNA samples overnight at 70°C. The solution was then treated with RNase A1 (Sigma) and T1 (Boehringer), and precipitated with 6 mol/l trichloroacetic acid according to Ekberg et al. [7]. Precipitates were collected on Whatman GF/C filters (Clifton, N.J., USA), which were then washed with 4% trichloroacetic acid in 5 mmol/l $Na_4P_2O_7$. The hybrid was solubilized in 1 ml Soluene 350 (Packard) and counted in a scintillation counter. To quantify the concentration of EGFr mRNA in the samples, the antisense probe was hybridized in a standard curve to known amounts of sense RNA (10–10000 amol). The abundance of EGFr mRNA in the unknown samples of TNA was expressed as fmol message/mg DNA. The specificity of this assay for EGFr mRNA has been shown earlier by others [7], using Northern blot and solution hybridization, and by Damber et al. [3] in an previous study from this laboratory using a RNA protection assay and solution hybridization.

Statistics

Values are expressed as medians with 25th and 75th percentiles. Comparisons between groups were performed using the Mann-Whitney *U*-test. A *P*-value of less than or equal to 0.05 was considered to be statistically significant.

Results

EGFr expression evaluated by binding capacity

A typical Scatchard analysis is shown in Fig. 1. In the 19 tumors a five fold increase in binding capacity could be observed, which was significantly increased compared with the normal kidney cortex ($P = 0.025$, Table 1). Diploid tumors had a significantly higher EGFr binding capacity than aneuploid tumors ($P = 0.001$). The EGFr

Table 1 EGFr binding capacity and mRNA expression in 19 kidney cortex and renal cell carcinomas in relation to clinical stage, grade and DNA ploidy. Median values are given with the 25th and 75th percentiles in parentheses

Tissue	No	mRNA (fmol/mg DNA) median		Binding capacity (fmol/mg protein) median	
Kidney cortex	19	11.0 (6–16)	$P<0.001$	150 (49–186)	$P=0.025$
Tumors	19	31.5 (22–45)		319 (131–490)	
Stage					
I	6	29.5 (28–32)	ns	711 (300–2810)	ns
II–III	6	108.0 (27–113)	ns	131 (69–217)	ns ($P=0.052$)
IV	7	41.0 (15–45)	ns	328 (314–490)	ns
Grade					
1–2	2	34.5 (32–37)		2324	
3	13	28.0 (18.5–46)	ns	396 (131–490)	ns
4	5	36.0 (24.5–77)		268 (170–329)	
Ploidy					
Diploid	9	37.0 (28–45)	ns	490 (219–2324)	$P=0.027$
Aneuploid	10	28.0 (18–42)		223 (96–407)	

binding capacities for the different grades and stages are shown in Table 1. There was no difference in tumor EGFr binding capacity between patients alive and patients dead of the disease.

EGFr mRNA

There was a significant overexpression of EGFr mRNA in tumors compared with kidney cortex tissue ($P<0.001$). Since there was no significant variation in expression of the EGFr gene among the 19 normal kidney cortex tissues, tumors were evaluated as overexpressed if the amounts of EGFr mRNA in the tumors were at least twice that of kidney cortex tissue. We found an overexpression in 11 of 19 tumors (58%). Although there was a numerical increase in mRNA values between the increasing grades and ploidy, we found no significant difference. No difference in mRNA levels between survivors and nonsurvivors could be shown.

There was a significant correlation between EGFr binding capacity and EGFr mRNA ($r=0.63$, $P=0.004$).

Discussion

The results of the present study show that renal cell carcinoma and normal kidney cortex tissues contain receptor proteins with affinity for EGF. Our findings showing an increased binding capacity and mRNA overexpression of EGFr in renal cell carcinoma compared with kidney cortex tissues from the same patient are in line with the results reported by other investigators [15, 24, 30].

A frequent cytogenetic change in renal cell carcinoma is the gain in chromosome 7 that may confer growth advantage to some malignant cells because of the mapping of the EGFr to this chromosome [17, 23]. However, these chromosomal alterations have been demonstrated to be present both in normal and tumor adjacent kidney cortex tissue [8, 9]. A study by Petrides and coworkers [22] showed underexpression of *proEGF* gene and overexpression of the genes for EGFr when compared with normal renal cortex tissue, which is in agreement with the present findings. *EGFr* gene amplification correlates with aggressiveness in several malignant tumors. In breast cancer 30% of breast tumors had amplification of the *EGFr*-related gene, *HER-2/neu* [27]. The presence of this amplification had greater prognostic value for relapse than most currently used factors including hormone receptor status and lymph node involvement. In one series of bladder cancer, EGFr expression measured by immunohistochemistry correlated with tumor recurrence, progression and overall survival. However, the incidence of gene amplification was only 1/29 [12]. In another study, an inverse relationship of *EGFr* and *HER-2/neu* gene expression was found in renal cell carcinoma compared with normal kidney tissue. With additional Southern blot analysis there was no indication of *EGF* gene amplification [29]. Ishhikawa et al. [15] found overexpression of the *EGFr* gene in 12 of 20 tumors, with the highest expression being found in the only case with geneamplification. In one study of 30 renal cell carcinomas, Gomella et al. [11] found increased expression of mRNA. However, there was no evidence of *EGFr* gene amplification and they concluded that amplification of this gene does not appear to be a common feature of renal cell carcinoma. In the present study, no obvious difference in EGFr expression could be demonstrated between the different grades and stages. Although we found a difference in EGFr binding capacity between diploid and aneuploid tumors, no differences in EGFr mRNA expression could be demonstrated.

The lack of *EGFr* gene amplification indicates that EGFr might not be involved in the malignant transformation of renal cell carcinoma. Our findings showing the absence of a clear relationship between tumor characteristics and *EGFr* expression support such a theory. The enhanced EGFr gene expression in renal cell carcinoma might be related to general cell growth advantages. Tumor-produced regulatory growth factors may play a role in the development of renal cell carcinoma [10]. It has

been suggested that the malignant transformation may be associated with the increased expression of tumor-produced growth factors and their receptors resulting in growth stimulation [13]. Enhanced expression of messenger RNA for EGFr and other growth factor related proteins in tumor relative to normal tissue have been reported in several neoplasms [4]. Many transformed cells produce the polypeptide transforming growth factor-alpha (TGF-α), which is structurally related to EGF and binds to the EGFr; high levels of TGF-α are consistently found in renal cell carcinoma cell lines and solid tumors [4]. Based on the present study and other reports, EGFr overexpression in renal cell carcinoma may be explained by a mechanism other than gene amplification to overproduce EGFr mRNA. The increased mRNA levels may be due to increased transcription or increased stability of the RNA.

It was concluded that EGFr is overexpressed in renal cell carcinoma when compared with normal kidney cortex tissue, both at the transcriptional and the protein level. However, no clear-cut relationship between EGFr expression and tumor characteristics was found. The expression of EGFr therefore appears not to be a useful marker for the characterization of renal cell carcinoma.

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