Inverse Relationship of Epidermal Growth Factor Receptor and HER2/neu Gene Expression in Human Renal Cell Carcinoma

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ABSTRACT

Expression of the oncogenes, epidermal growth factor (EGF) receptor, HER2/neu, c-myc, and c-fos, in renal cell carcinoma and corresponding nonneoplastic kidney tissue of 30 patients has been analyzed by Northern blot analysis. In renal cell carcinoma an inverse relationship of EGF receptor and HER2/neu gene expression was detected, with high expression of the EGF receptor gene in 22 of 30 (73%) cases and low expression of the HER2/neu gene in 28 of 30 (93%) cases. Furthermore, altered expression of the oncogenes c-myc and c-fos was detected in renal cell carcinoma, which appears to be related to the tumor grade of malignancy.

Additional Southern blot analysis of six renal cell carcinomas gave no indication of chromosomal rearrangement events or gene amplification.

INTRODUCTION

Renal cell carcinoma is the most common malignancy of the kidney in adults (1, 2). The development of renal cell carcinoma begins within the renal parenchyma. The tissue of origin involves the proximal renal tubules as demonstrated by electron microscopic studies (3). Two different forms of renal cell carcinoma have been described, often associated with a nonrandom chromosomal abnormality of chromosome 3: a typical sporadic form and a less common (1-2%) hereditary form (4-7).

The association of specific cellular oncogene expression with tumor initiation, promotion, and progression has been examined in various human malignancies, including lymphoma, leukemia, and carcinomas of the thyroid gland, breast, and colon (8-11). Previous studies suggest amplification and/or overexpression of the gene for the EGF receptor in human malignancies (9, 12, 13). The EGF receptor gene encodes a 170,000 membrane-bound glycoprotein with intracellular tyrosine kinase activity and an extracellular binding site for EGF, transforming growth factor alpha alpha (14). The product of the HER2/neu gene is a receptor-like glycoprotein with a cytoplasmic tyrosine kinase domain (15, 16); in contrast to the EGF receptor, a ligand has not yet been identified. The biological role of the HER2/neu gene product is still unknown, but its sequence homology with EGF receptor of approximately 50% overall (16) suggests that the function of these two oncogenes may be related. The EGF receptor gene is on chromosome 7p13-p12; the HER2/neu gene is on 17q11.2-q12 (17). An increased expression of the oncogenes c-myc and c-fos has been reported for a wide spectrum of human malignancies (8), suggesting that the oncogenes c-myc and c-fos are crucial factors in the regulation of cellular proliferation and differentiation. Recent studies have shown that both genes encode proteins related to known transcription factors (20).

In order to examine the relevance of these four oncogenes to cancers of the urogenital tract, we have analyzed their expression in 30 unilateral renal cell carcinomas and corresponding normal nonneoplastic kidney tissues using the Northern and Southern blot analysis techniques.

MATERIALS AND METHODS

Tissue. Tissue samples from renal cell carcinoma and corresponding normal nonneoplastic kidney were obtained from 30 previously untreated patients of the Urologische Klinik, University of Düsseldorf, Federal Republic of Germany. Tumor specimens were classified according to the International Union Against Cancer recommended “TNM-Classification for Renal Carcinoma” (21). The grading of malignancy was mainly performed by the same pathologist. The tissue specimens were frozen in liquid nitrogen immediately after radical nephrectomy and stored at -70°C until they were analyzed. Furthermore, tissue samples from term placenta and from a kidney without any pathological alteration were examined.

Hybridization Probes. The following human DNA hybridization probes were used in the analysis of cellular oncogene expression: the 1.8-kilobase EcoRI cDNA fragment of the human EGF receptor gene (clone HER-A64-1) (22) and the 1.6-kilobase EcoRI cDNA fragment of the human HER2/neu gene (clone HER-2-436-2) (15) (both of these cDNA probes were kindly provided by Professor Dr. A. Ullrich, Martinsried, Federal Republic of Germany); the genomic 3.1-kilobase Xhol-NcoI fragment of the c-fos oncogene (23, 24) excised from pSP65 and the genomic 1.5-kilobase SstI fragment of the c-myc oncogene (25, 26) cloned in pSP64 (both oncogene probes were obtained from Amersham Buchler, Braunschweig, Federal Republic of Germany); the 3.6-kilobase HindIII fragment of the β-actin-related pseudogene HßAc-γ2 (27) separated from pB322 (kindly provided by Dr. W. Schulz, Institut für Physiologische Chemie I, University of Düsseldorf, Federal Republic of Germany).

Isolation of DNA and RNA. Genomic DNA and total cellular RNA were isolated from the same tissue samples by means of a combination of the methods of Chirgwin et al. (28) and Davis et al. (29). Concentration and purity were determined by absorbance at 260 and 280 nm according to the method of Berger (30). The integrity of total RNA was estimated by observing the intensities of ethidium bromide-stained RNA bands after fractionation in 1.0% (w/v) agarose gel containing 2.2 M formaldehyde. Poly(A)°RNA was selected by oligo(dT)immobilized acid cellulose chromatography with a modified method of Davis et al. (29).

Northern Blot Analysis. Five μg denatured poly(A)°RNA were electrophoresed in 1.0% (w/v) agarose gel containing 2.2 M formaldehyde. RNA was then transferred to a nylon membrane (NY 13 N from Schleicher and Schuell, Dassel, Federal Republic of Germany) by blotting overnight in 20x SSC and fixed by incubation at 80°C for 1 h. The sizes of the transcripts were determined by using 10 μg of the RNA molecular size marker I (Boehringer Mannheim, Mannheim, Federal Republic of Germany) loaded to each agarose gel. Membranes were prehybridized for 3–5 h at 42°C in 50% (v/v) deionized formamide, 5x standard saline phosphate EDTA (0.15 M sodium chloride-0.01 M sodium citrate, pH 7.0), 0.5% SDS, 0.15 M sodium chloride-0.015 M sodium citrate, 0.5% SDS, 0.15 M sodium chloride-0.015 M sodium citrate, pH 7.0, 0.15 M sodium chloride-0.015 M sodium citrate, pH 7.0; SDS, sodium dodecyl sulfate; T/C, tumor/control ratio.
sodium dihydrogen phosphate-0.001 M EDTA, pH 7.4), 5× Denhardt's solution [0.02% (w/v) Ficoll-0.02% (w/v) polyvinylpyrrolidone-0.02% (w/v) bovine serum albumin], 0.1% (w/v) SDS, and 0.2 mg/ml sonicated, heat-denatured herring sperm DNA. Hybridization with 3P-labeled DNA probes was carried out overnight at 42°C in 50% (v/v) deionized formamide, 5× SSC, 1× Denhardt's solution, 0.1% (w/v) SDS, and 0.2 mg/ml sonicated, denatured herring sperm DNA. All DNA probes were derived from plasmids and 3P labeled to a specific activity of 2-5 × 10^6 cpm/µg DNA by using the oligo-labeling technique (31). After hybridization, the membranes were washed for 5 min in 5× SSC, 1.0% (w/v) SDS at room temperature, followed by two washes in 1× SSC, 1.0% (w/v) SDS for 15 min at 37°C, and finally in 1× SSC, 1.0% (w/v) SDS for 15 min at 50°C. Radioactivity was detected with Kodak X-OMAT film at -70°C with an intensifying screen for 16-42 h. Hybridization signals of specific transcripts were further quantified by densitometric scanning with an LKB 2202 ultrascan laser densitometer. After hybridization and autoradiography, the membranes were stripped by washing in 5 mM Tris-HCl, pH 8.0-0.2 mM EDTA-0.1× Denhardt's solution-0.05% (w/v) sodium pyrophosphate for 1 h at 65°C.

Southern Blot Analysis. Ten µg of high molecular weight DNA were digested with EcoRI, fractionated on a 0.5% (w/v) agarose gel and blotted onto nylon membrane (Gene Screen Plus, NEN, Boston, MA) according to the Southern method (32). The high molecular weight marker (BRL, Gaithersburg, MD) and EcoRI/HindIII-digested R-DNA were used as molecular weight markers. Hybridization to 3P-labeled DNA probes was performed by the method of Church and Gilbert (33). After hybridization the membranes were washed three times in 200 HIM sodium phosphate buffer, pH 7.2, and 1.0% (w/v) SDS for 30 min at 65°C, followed by a final wash in 50 mM sodium phosphate puffer, pH 7.2, and 1.0% (w/v) SDS for 20 min at 65°C. The membranes were then exposed to Kodak X-OMAT film at -70°C with an intensifying screen for at least 3 days. For reuse the membranes were washed in 2 mM EDTA, pH 8.0, and 0.1% (w/v) SDS for 1 h at 80°C.

RESULTS

Expression of the oncogenes, EGF receptor, HER2/neu, c-myc, and c-fos in 30 renal cell carcinomas and adjacent non-neoplastic kidney tissues was examined with Northern blot analysis. Poly(A+)RNA was isolated as described in “Materials and Methods”. Poly(A+)RNA was also prepared from tissues of term placenta and normal kidney from patients with no known renal disease. Gene expression was assessed by specific oncogene transcripts identified by autoradiography. The amount of mRNA transcripts was analyzed by densitometric measurement, and the relative amount of total RNA was quantified by membrane rehybridization with the β-actin-related pseudogene H/Ac-β2 (27) to detect β-actin gene expression as an internal standard. The ratio between oncogene expression in renal cell carcinoma relative to that in corresponding non-neoplastic kidney tissue was normalized to β-actin gene expression and is indicated as the T/C ratio.

Increased Expression of the EGF Receptor Gene. In most cases increased expression of the EGF receptor gene was detected in renal cell carcinoma. When analyzed with Northern blot hybridization, 27 of 30 (90%) tumors as compared to only 19 of 30 (63%) adjacent nonneoplastic kidney tissues displayed an EGF receptor-specific 10.5-kilobase transcript (Fig. 1). Only in a few cases was a 5.8-kilobase transcript of the EGF receptor gene also observed. Unlike in term placenta with almost similar expression of the 10.5- and 5.8-kilobase transcripts, renal cell

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Fig. 1. Northern blot analysis of EGF receptor and HER2/neu RNA in human renal cell carcinoma (T), corresponding nonneoplastic kidney tissue (C), term placenta (P), and tissue from nonpathological kidney (K). Each lane contained 5 µg poly(A+)RNA size fractionated in 1.0% formaldehyde agarose gel. Electrophoresis, transfer onto nylon membrane, and hybridization were performed as described in “Material and Methods.” Our data reveal an inverse relationship of EGF receptor and HER2/neu gene expression in renal cell carcinoma. In nonpathological kidney tissue, low expression of the EGF receptor gene is accompanied by high expression of the HER2/neu gene. In renal cell carcinoma, however, expression of the EGF receptor is high and expression of the HER2/neu gene is low compared to corresponding nonneoplastic kidney tissue. The oncogene expression was quantified by densitometric measurement and normalized by β-actin gene expression being used as internal standard. The RNA transcript sizes were determined by the RNA molecular size marker I from Boehringer Mannheim. The increased EGF receptor gene expression in renal cell carcinoma is indicated by the 10.5-kilobase (kb) transcript and the HER2/neu gene expression by the 4.8-kilobase transcript. In case 287 only an altered HER2/neu gene transcript of 2.1 kilobases was detected. The case numbers at the top of the figure refer to the case numbers listed in Table 1. Exposure time was 24 h.

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ONCOGENE EXPRESSION IN RENAL CELL CARCINOMA

Table I Expression of oncogenes in 30 human renal cell carcinomas

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*Histopathologically determined grade of malignancy according to the UICC recommended "TNM-Classification for Renal Carcinoma."

Grade Specificity of c-myc and c-fos Gene Expression. Hybridization of the samples to the c-myc probe revealed the presence of a 2.3-kilobase transcript (Fig. 2). In 19 of 30 (63%) cases expression of the c-myc gene was found in tumor tissue as opposed to expression in only 13 of 30 (43%) nonneoplastic kidney tissue samples. An increased level of c-myc gene expression was detected in tumor tissue in 13 cases, the T/C ratio being between 2 and 5.5 in six cases; in one case a 72-fold overexpression of c-myc and an additional 3.0-kilobase transcript, whereas no c-fos gene expression is detected, β-Actin expression was used as internal standard. All membranes were exposed for 36 h.

In contrast to c-myc, the c-fos gene was found to have a lower expression in 16 of 22 hybridized samples indicated by a 2.2-kilobase transcript (Fig. 2). In seven cases c-fos gene-specific transcripts were only detected in nonneoplastic kidney tissue. In 9 cases the T/C ratio varied between 0.15 and 0.75. In one case expression of the c-fos gene showed similar levels. In five cases, increased expression was detectable, whereas in 8 cases specific transcripts were detected neither in tumor nor in nonneoplastic kidney tissue (Table 1).
The levels of c-myc and c-fos gene expression were correlated to the grade of malignancy as determined by histopathological criteria (21). With increasing malignancy grade, c-myc was nearly consistently overexpressed in renal cell carcinoma, whereas c-fos gene expression levels were decreased (Table 2). Expression of the EGF receptor and HER2/neu genes showed no specific pattern of correlation with the grade of malignancy.

### Southern Blot Analysis

Changed oncogene expression can often be correlated with chromosomal rearrangement or gene amplification (34). In order to detect possible chromosomal rearrangement events or gene amplification, we isolated high molecular weight DNA from six renal cell carcinomas, corresponding nonneoplastic kidney tissues, and term placentas. Comparative Southern blot analysis with EcoRI-digested DNA did not reveal abnormal digestion patterns or gene amplification. Sample 28T is a case in point: characterized by substantial rearrangement events or gene amplification, we isolated high molecular weight DNA from six renal cell carcinomas, corresponding nonneoplastic kidney tissues, and term placentas. Comparative Southern blot analysis gave no indication of chromosomal rearrangement or gene amplification. Especially in case 28T, which is characterized by a substantially altered c-myc and HER2/neu gene expression, no amplified or rearranged oncogenes were detected. The sizes of the oncogene-specific DNA signals are indicated in kilobases (kb). Membranes were exposed for at least 72 h.

### DISCUSSION

EGF Receptor and HER2/neu Gene Expression. The oncogenes, EGF receptor, HER2/neu, c-myc, and c-fos, have been associated with tumorigenesis (8-13, 18, 19). We have studied the expression of these four oncogenes in 30 human renal cell carcinomas. In general, our data show an inverse relationship of EGF receptor and HER2/neu gene expression. In normal nonpathological kidney tissue low expression of the EGF receptor gene is contrasted to high expression of the HER2/neu gene (Fig. 1). In renal cell carcinoma, the pattern is reversed to high expression of the EGF receptor and low expression of the HER2/neu gene. An enhanced expression of the EGF receptor gene in renal cell carcinoma has been observed by several groups (35, 36, 37), whereas the determined expression of the HER2/neu gene in kidney tissue found here seems to contradict the results of Yao et al. (35), who did not detect any expression of the HER2/neu gene in renal cell carcinoma or in adjacent nonneoplastic kidney tissue from Japanese patients. Whether these results suggest a HER2/neu gene expression in a population-specific manner in adult kidney tissue remains to be elucidated.

Since several groups detected increased expression of EGF receptor and/or transforming growth factor α in renal cell carcinoma by Northern blot analysis and immunohistochemistry (35-38), an autocrine mechanism involved in tumor cell proliferation and differentiation seems to be possible, probably by activation of the tyrosine kinase activity of EGF receptor.

In term placenta the EGF receptor-specific 10.5- and 5.8-kilobase transcripts were expressed at similar levels, whereas in renal cell carcinoma and normal kidney tissue a higher level of the 10.5-kilobase transcript was detected (Fig. 1). It is uncertain whether the increased expression of the 10.5-kilobase EGF

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*Histopathologically determined grade of malignancy according to the UICC recommended "TNM-Classification for Renal Carcinoma."

*†, increased expression in renal cell carcinoma (RCC) compared to nonneoplastic kidney tissue; 0, equal expression in RCC and corresponding nonneoplastic kidney tissue; —, decreased expression in RCC compared to nonneoplastic kidney tissue; N, no detectable expression in RCC and corresponding nonneoplastic kidney tissue.
ONCOGENE EXPRESSION IN RENAL CELL CARCINOMA

with increasing malignancy, since tumor progression and in conclusion: c-fos gene expression in renal cell carcinoma is expression is due to the loss of one or even both of the c-fos qter] (5), which is indeed the exact location of the c-fos gene. Sporadic renal cell carcinoma to chromosome 14 [del(14)(q22-q31)] (46). Recent cytogenetic studies have pinpointed one of the most common chromosomal aberrations associated with sporadic renal cell carcinoma to chromosome 14 [del(14)(q22- qter)] (5), which is indeed the exact location of the c-fos gene. It is, therefore, quite possible that the reduced level of c-fos expression is due to the loss of one or even both of the c-fos alleles, although our experiments gave no indication of such chromosomal rearrangement events.

The loss of one or possibly both c-fos alleles, however, could be the explanation for the further decrease of c-fos expression with increasing malignancy, since tumor progression and increased malignancy are factors responsible for decreased genetic stability (34, 47). In order to be able to examine the structural condition of chromosome 14 in more detail it would be necessary to analyze a greater number of tumor DNA samples using the widest possible range of restriction endonucleases.

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