Aberrant Expression of Epidermal Growth Factor Receptor and HER-2 (erbB-2) Messenger RNAs in Human Renal Cancers

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ABSTRACT

Amplification, rearrangement, or overexpression of the gene for the epidermal growth factor receptor (EGFR) occurs in certain types of human neoplasia. We investigated EGFR gene structure and measured EGFR mRNA levels in human renal tumor biopsies. Seventeen renal tumors (13 renal cell carcinomas [RCCs], two Wilms' tumors, one osteosarcoma, and one metastatic ganglioneuroblastoma) and their corresponding normal kidney tissues were examined for EGFR gene structural integrity by Southern blot hybridization. Twelve of these tumors (including 11 RCCs) were examined for EGFR mRNA expression levels by RNA blot hybridization. The EGFR gene was rearranged in one of 13 (8%) of the RCC specimens examined and was highly amplified in the ganglioneuroblastoma. The overall frequency of EGFR gene structure alterations in this series of renal tumors was 12%. Nine of 11 RCC specimens (82%) exhibited markedly elevated EGFR mRNA levels (approximately 2- to 6-fold). In contrast, expression of the EGFR-related protooncogene HER-2 (erbB-2) was found to be decreased in 11 RCCs and one Wilms' tumor; HER-2 gene structure, however, appeared normal in all specimens. These results indicate that overexpression of EGFR mRNA, probably due to changes in gene regulation, and underexpression of HER-2 mRNA are characteristic features of human RCC.

INTRODUCTION

The EGFR is the natural cell surface receptor for EGF and for TGF-α. The EGFR is a M, 170,000 glycoprotein with three distinct functional and structural domains: a 621-amino acid extracellular domain involved in ligand binding; a 542-amino acid intracellular cytoplasmic domain, which possesses an intrinsic tyrosine kinase activity; and a small region that spans the plasma membrane (1). The binding of EGF or TGF-α to the EGFR activates the tyrosine kinase activity and initiates a signal transduction process that results in ligand-induced responses in the target cell (2).

An altered, truncated form of the EGFR gene (erbB-1) is the transforming gene of avian erythroblastosis virus (3), indicating that aberrant expression or mutation of the EGFR gene can lead to neoplastic transformation. Studies of human biopsy specimens have demonstrated abnormally high expression of the EGFR in a number of human epithelial malignancies, including breast, vulval, head and neck, genitourinary, and lung carcinomas and certain brain tumors (4-13). EGFR elevation has been most consistently demonstrated for many types of squamous cell neoplasia (5, 14). In certain cases, EGFR gene amplification or rearrangement has been observed (1, 14-16).

In this study we have examined tissue from a series of 17 renal tumor biopsies, including one metastatic ganglioneuroblastoma and two Wilms' tumor specimens, for EGFR gene structure and have measured EGFR mRNA levels in a subset of these tumor specimens. The EGFR-related protooncogene, HER-2 (also referred to as erbB-2 and c-neu; hereafter HER-2) (18, 19), was examined in the same fashion in these specimens.

MATERIALS AND METHODS

Tumor Specimens. Tumor and normal tissues were obtained immediately following surgical removal at The University of Texas M. D. Anderson Cancer Center, Houston, TX. Histological diagnosis and nuclear grade for all specimens were provided by the Department of Pathology, M. D. Anderson Cancer Center.

DNA and RNA Isolation. Total genomic DNA was prepared from tissue samples according to the protocol of Davis et al. (20), with the following modifications. Samples were treated for 30 min with 20 µg/ml of DNase-free pancreatic RNase A and for 30 min with 20 µg/ml of proteinase K prior to phenol and chloroform extraction. RNA was isolated from tissue samples by the guanidinium isothiocyanate extraction method as described by Chirgwin et al. (21). All tissue samples were stored at −80°C prior to nucleic acid extractions.

Southern Blotting and Hybridization. Genomic DNA concentrations were determined by spectrophotometric measurement of absorbance. Restriction endonuclease digestion was performed according to the specifications of the manufacturer (Boehringer Mannheim, Indianapolis, IN). Digested DNAs were electrophoresed through 0.8% agarose gels. Gels were stained with ethidium bromide, photographed, and exposed briefly to ultraviolet illumination. The gels were washed with 1.5 M NaCl/0.5 M NaOH, neutralized in 1 M Tris/3.0 M NaCl, and blotted onto nylon membranes (Zetaprobe; Bio-Rad, Richmond, CA); blotting buffer was 10x standard saline citrate. Southern blots were baked at 80°C for 1 h after transfer. DNA probes were labeled with 32P by the random oligonucleotide method using the Multiprime labeling kit (Amersham, Arlington Heights, IL). Probe specific activities were >1 x 109 cpn/Mg. Nylon membranes were hybridized overnight at 65°C in 1 M NaCl, 10% dextran sulfate, and 1% sodium dodecyl sulfate with 1 x 106 cpn/ml of probe. Washes were performed under stringent conditions (final wash, 0.5 x standard saline citrate at 65°C). Autoradiography of the hybridized filters was performed by exposure to Kodak XAR film at −80°C.

RNA Dot Blots and Hybridization. RNA dot blotting was accomplished by denaturing RNA in 18% formaldehyde/50% formamide at 65°C and applying the samples to Zetaprobe membranes, using a Schleicher and Schuell blotting manifold. Membranes were baked at 80°C for 1 h after blotting. RNA hybridization was subsequently carried out at 65°C in 1 M NaCl, 10% dextran sulfate, and 1% sodium dodecyl sulfate with 1 x 106 cpn/ml of probe. Washes were performed under stringent conditions. Autoradiography was performed by exposure to Kodak XAR film at −80°C. Autoradiograms were analyzed by densitometric scanning, using a Bio-Rad Model 620 video densitometer and 1-D Analyst software (Bio-Rad).

Hybridization Probes. The 800-base pair EGFR cDNA clone p64-3 was used for both RNA and DNA blot hybridization experiments (22). This probe recognizes approximately 20 kilobases of the 118-kilobase human EGFR gene locus and corresponds to the transforming tyrosine kinase gene.
kinase domain of v-erbB (3). The HER-2 probe was a 3-kilobase KpnI- HindIII fragment from the pCER-204 plasmid (19) corresponding to extracellular, transmembrane, and some intracellular HER-2 regions. These two probes represent nonoverlapping domains of the common linear structure of the EGFR and HER-2 mRNAs. Both probes were kindly provided by Dr. Mien-Chie Hung, Department of Tumor Biology, M. D. Anderson Cancer Center. The β-actin cDNA probe was kindly provided by Dr. Timothy C. Thompson, Department of Urology, Baylor College of Medicine, Houston, TX.

RESULTS

In the following experiments we have examined a series of human renal tumors and clinically normal kidney samples from the same patient in search of structural abnormalities in the EGFR and HER-2 genes. Additionally, using tumor samples from which intact RNA could be obtained in sufficient quantity, we have measured EGFR, HER-2, and β-actin mRNA transcript levels relative to the levels of matched control kidney samples. Clinical data and histological evaluation of the tumors examined are presented in Table 1. Thirteen RCCs, 2 Wilms’ tumors, one oncocytoma, and one ganglioneuroblastoma were examined for EGFR and HER-2 gene integrity; 11 RCC specimens and one Wilms’ tumor specimen were examined for EGFR and HER-2 mRNA expression.

EGFR and HER-2 Gene Structure. Chromosomal DNA was extracted from tumors and normal kidney specimens by conventional preparative procedures and hybridized with an EGFR cDNA probe, according to standard methods for Southern blot analysis (see “Materials and Methods”). The results are summarized in Table 1. Two distinct EGFR gene abnormalities were detected on Southern blots in two separate tumor specimens (Fig. 1). An RCC specimen was found to contain an abnormal EGFR allelic fragment that was not present in the normal kidney specimen from the same patient or in any other DNA specimen we examined. A second tumor, a metastatic ganglioneuroblastoma, was found to possess highly amplified (≥10-fold) EGFR gene sequences in comparison to normal kidney DNA from the same patient and to normal and tumor DNA from the remaining tumor samples. Fifteen other tumors and a pyelonephritis specimen tested were found to possess normal EGFR patterns on Southern blots in the gene regions homologous to the cDNA probe. The frequency of EGFR abnormalities in the RCC specimens was 8% (1 of 13), and the overall frequency of abnormality in this group of tumors was 12% (2 of 17). No kidney samples designated as clinically normal possessed aberrant EGFR gene hybridization patterns. All DNA samples were also hybridized with a cDNA probe for the HER-2 gene, a structurally related but distinct protooncogene. No HER-2 gene abnormalities were detected in any of the specimens (Table 1).

EGFR, HER-2, and β-Actin mRNA Expression. Relative levels of EGFR, HER-2, and β-actin mRNAs were determined for 11 RCC specimens and one Wilms’ tumor specimen. Total cellular RNA was isolated from renal tumors and from clinically normal kidney specimens from the same patient as internal controls. RNA was purified from tissues by conventional preparative procedures, and the integrity of the RNA preparations was checked by agarose gel electrophoresis followed by ethidium bromide staining (see “Materials and Methods”). Samples from 12 tumors yielded sufficient intact RNA (as evaluated by 28S ribosomal RNA band integrity) to be assayed for mRNA expression. In the following experiments, tumor and normal tissues from the same patient were matched against each other on the same hybridization filters, with the exception of Tumor K (normal kidney tissue was not available from this patient). An estimate of normal expression for Tumor K was made by averaging densitometric data of four normal samples probed on the same filters with Tumor K RNA. Serial dilutions (20 μg to 1.25 μg) of tumor and matched normal kidney RNAs were blotted directly onto nylon hybridization membranes and probed initially under stringent hybridization conditions with EGFR cDNA probe.

Representative samples of the results obtained are shown in Fig. 2, where we demonstrate significantly elevated EGFR mRNA expression in six separate tumor specimens. Messenger RNA expression levels for all specimens were quantitated by densitometric scanning of the autoradiograms, and the data are summarized in Table 2. We find substantially elevated levels (>2-fold) of EGFR mRNA transcripts in 9 of 11 (82%) of the RCC tumor specimens. The range of elevation is approximately 2- to 6-fold. EGFR mRNA was detectable in all normal kidney samples by this assay method. The single Wilms’ tumor showed normal EGFR levels. Therefore the overall frequency of EGFR

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mRNA elevation in the renal tumors examined was 75%. The overall change observed is highly significant ($P = 0.001$). Usable RNA was not obtainable from the metastatic neuroblastoma specimen with an amplified EGFR gene, and we therefore could not make an expression determination for this specimen.

Relative levels of expression of the HER-2 and β-actin genes were also determined by serial rehybridization of membranes hybridized previously with the EGFR probe. The EGFR and HER-2 probes correspond to nonoverlapping, non-cross-hybridizing domains of the common linear structure of the EGFR and HER-2 mRNAs. HER-2 mRNA transcripts were detectable in all specimens tested. HER-2 mRNA levels were lower in all 12 tumor specimens than in normal controls, whereas β-actin expression was unchanged (Fig. 3; Table 2). The average relative value of HER-2 (tumor/normal = 0.55) represented a diminution in mRNA expression of approximately one half. This degree of change is near the limit of resolution of this methodology; however, overall HER-2 expression in this group of tumor specimens departs significantly from randomness ($P < 0.001$), while β-actin expression does not ($P = 0.69$; mean = 1.01).

**DISCUSSION**

We have examined the structure and expression of the EGFR and HER-2 genes in a series of human renal tumor biopsy specimens. Aberrant EGFR genes were identified in a ganglion-neuroblastoma (gene amplification) and an RCC specimen (rearranged allele). This represents an EGFR structural aberration frequency of 12%. No kidney specimens designated as normal showed aberrant Southern hybridization patterns. Although we could not reliably measure mRNA levels from the metastatic ganglioneuroblastoma specimen, it is noteworthy that this tumor was found to possess an amplified EGFR gene. A correlation between invasiveness and EGFR expression has been previously determined for certain genitourinary (bladder) tumors (23).
showed one of the highest relative levels of EGFR overexpression.

Our results suggest that elevation of EGFR mRNA levels occurs frequently in renal cell carcinomas. The detection of two EGFR gene abnormalities in this series of tumors would suggest that overexpression of EGFR mRNA probably results from aberrant gene regulation in neoplastic renal cells. To our knowledge there has only been one previous report examining EGFR expression in a significant number (i.e., 10 or more) of human renal tumors. Yao et al. (13) recently published EGFR mRNA expression data for a separate group of human RCCs. These investigators report an overall incidence of EGFR mRNA elevation in 47% (7 of 15) of the tumors they tested, a result that is not significantly different from our own. The relative extent of change they reported (approximately 2- to 7-fold) was also consistent with our data. Six tumors showing EGFR elevation tested negatively for alterations in EGFR gene structure by Southern hybridization. Yao et al. did not, however, present HER-2 mRNA expression data; they report that the transcript was undetectable using their assay conditions. This observation is not consistent with our data nor with published data on expression of c-neu (the rat HER-2 counterpart) in the adult kidney. The HER-2 (c-neu) gene product, p185, is expressed in a stage- and tissue-specific manner during development in rodents and is constitutively expressed at substantial levels in certain adult epithelial cell types, including kidney (24). Our results indicate that aberrant expression (underexpression) of the HER-2 gene is also a frequent event in human RCC.

Underexpression of HER-2 in RCC may be a reflection of a phenotypic dedifferentiation of kidney epithelium associated with neoplasia and/or aberrant regulation of the HER-2 gene. Another possibility, that underexpression is a consequence of a significant dilution of epithelial cell types with mesenchymal cell types in the tumor mass, is considerably less likely; the epithelial nature of the RCC specimens has been clearly demonstrated by histopathological analysis. Furthermore, both the EGFR and the HER-2 proteins are predominantly expressed in epithelial cell types, and the changes we have observed occur in opposing direction; dilution by stromal cell types probably cannot account for both changes simultaneously. Recently, underexpression of the HER-2 p185 in cell lines derived from anaplastic colonic tumors has been reported (25).

Overexpression and amplification of the HER-2 gene are a common feature of aggressive forms of breast cancer in humans (26, 27). Slamon et al. (27) have recently presented evidence for a similar involvement of HER-2 expression in human ovarian cancers. Because the renal tumor specimens we have analyzed are primarily of high stage and grade (see Table 1), our data appear to exclude the possibility of a similar frequency of HER-2 overexpression in human RCC. Consistent with this conclusion, we did not detect HER-2 gene abnormalities in 17 tumor specimens examined, while EGFR gene alterations were detected.

The EGFR and the HER-2 proteins are involved in growth regulation by operating as transducers of signals from the extracellular to the intracellular compartments. The presumptive ligand for the HER-2 receptor has not yet been discovered; however, the EGFR is known to be a natural receptor for TGF-α as well as EGF. Cultured cells transformed by RNA and DNA tumor viruses have been found to express high levels of TGF-α (28). Derynck et al. (12) have demonstrated that elevation of EGFR mRNA frequently is accompanied by a parallel increase in TGF-α and TGF-β gene expression in a variety of human tumors and cell lines, pointing to the possible involvement of these growth factors in tumor progression. One of the consequences of ligand binding to the EGFR can be phosphorylation of the HER-2 protein, p185 (29). Therefore the degree to which these receptors interact to influence each others’ respective signal transduction pathway(s) in transformed cells remains to be determined. The data we have presented in this paper suggest that the EGF/TGF-α-response pathways are significantly perturbed or disrupted in human renal neoplasia. This perturbation may be a contributing factor to the expression of the malignant phenotype in renal tumor cells.

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REFERENCES


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