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Human tumor cell lines with EGF receptor gene amplification in the absence of aberrant sized mRNAs

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ABSTRACT

A survey of human tumor cell lines for increased PDGF or EGF receptors identified 5 lines which bind from 6 to 13 times more EGF than normal human fibroblasts. Immunoprecipitation analysis links the elevated binding activity to increased quantities of the EGF receptor protein. EGF receptor gene amplification was detected in 2 of the cell lines. No evidence for EGF receptor gene rearrangements was found at the level of DNA or RNA structure. The results suggest that elevated levels of EGF receptor can be associated with at least three distinct mechanisms. These include gene amplification accompanied by rearrangement, gene amplification without accompanied alteration of mRNA transcripts, and extensive expression without gene amplification.

INTRODUCTION

Recent discoveries have provided evidence that cellular growth control pathways may be disturbed in the neoplastic cell. Two viral oncogenes have been shown to encode proteins which are homologous to important effector molecules in regulation of normal cellular growth. The transforming gene of the simian sarcoma virus encodes a protein highly homologous to platelet-derived growth factor (PDGF) chain 2 (1-3). The protein product of this transforming gene bears many common structural and functional similarities to biologically normal PDGF. The transforming gene of the avian erythroblastosis virus is derived from the epidermal growth factor (EGF) receptor gene but has been structurally altered so as to encode a substantially truncated product (4). Although it is not yet known what, if any, function either proto-oncogene may contribute to the neoplastic process affecting human cells, the recent findings have provided the opportunity to compare functional assays for components of the growth control system with investigation of genetic alterations that may exist in gene structure that affect their corresponding messenger RNAs and proteins.

Recent studies have identified alterations in the cellular EGF receptor gene in the epidermoid cell carcinoma cell line A431 (5-8) and in some human

glioblastomas (9). In each case, the gene was found to be amplified and aberrant mRNAs were detected. In addition, other studies have shown that extensive expression of EGF receptor need not be accompanied by gross genetic change in human tumor cell lines (10). In this report, we study several human tumor cell lines which display high levels of cell surface EGF-binding activity. We identify cases of receptor gene amplification in the absence of aberrant sized mRNAs. Taken together, these results indicate that several molecular mechanisms can be associated with the high level expression of the EGF receptor in certain tumor cells.

MATERIALS AND METHODS

EGF binding assays

Cells (5 x 10^5) were plated in 35 mm dishes and grown overnight in media containing 10% serum. On the day of assay, total cellular protein was assayed from 2 plates. To assay total EGF binding, duplicate plates of cells were washed twice with 2 ml of binding buffer (Meloy Labs), incubated with 5 ng/ml ^{125}I EGF in 2 ml buffer at 22°C for 1 hr, washed three times with 2 ml binding buffer at 4°C, and finally lysed in 1 ml 0.1 M NaOH for counting. Nonspecific binding was assayed on a single plate of cells in the same way except that the incubation also included 10 {g/ml of non-radioactive EGF. Specific binding was calculated as the average total binding less the nonspecific binding.

Immunoprecipitation of EGF_r protein

Cells plated two days earlier at a concentration of 3 x 10^6/100 mm dish were incubated with 10 ml methionine-free Dulbecco's modified Eagle's medium (DMEM met-) for 30 min at 37°C, washed with 10 ml DMEM met- and metabolically labeled for 6 h with 300 {Ci [^{35}S]-methionine/ml DMEM met-. Following mechanical cell disruption, subcellular fractionation of cell lysates was done by high-speed centrifugation at 100,000 G. The pelleted membranes were dissolved in Staph A buffer (1% Triton x-100, 0.1% SDS, 0.5% sodium-deoxycholate, 0.1 M NaCl, 10 mM phosphate buffer, pH 7.4 containing 1.5 mM PMSF and 100 kallikrein inactivator units of Aprotinin/ml).

2 x 10^7 acid precipitable CPM/lane were incubated with excess of monoclonal EGF_r antibody (kindly provided by B. Hampar) for 45 min at 4°C. The receptor-antibody complex was adsorbed to 10 mg preswollen and prewashed protein A sepharose beads for 30 mins. at 4°C under shaking. After three washes in 1 ml Staph A buffer, the immune complex was boiled for 4 min. in the presence of β-mercaptoethanol. SDS polyacrylamide gel electrophoresis was performed in 6-16% polyacylamide gradient gels. Following fluorography with

Enhance (NEN) the slab gels were dried and exposed to x-ray at 70°C for 1 or 2 days.

DNA blotting

DNAs (15 {g) were restricted with Eco RI (New England Biolabs) and subjected to electrophoresis in 0.8% agarose gels. DNAs were transferred to nitrocellulose membranes (Schleicher and Schuell) by the method of Southern (11), using ammonium acetate as a convectant. The DNA dot blot analysis was conducted by making a 2-fold dilution series of total genomic DNA (which had been cleaved with Eco RI), denaturing the DNA with 0.1 M NaOH and neutralizing with 1 M NH_4Ac. DNA was applied immediately to nitrocellulose using a dot blot manifold (Schleicher and Schuell).

RNA blotting

Total cellular RNAs were isolated using the guanidine HCl method (12). Samples (25 {g) were denatured and subjected to electrophoresis in 0.7% agarose gels containing 0.36 M formaldehyde (13). Ribosomal RNAs were detected by ethidium bromide staining. RNA was transferred to nitrocellulose following mild alkali degradation (50 mM NaOH, 22°C for 30 min) using a solution of 1.5 M NaCl and 150 mM Na citrate as a convectant.

Hybridization

The EGF receptor probe consisted of isolated 2.4 kb cDNA removed by Cla I digestion from plasmid PE7 (7). Each probe was nick-translated to over 5×10^8 cpm/{g using $\alpha^{32}P$ dCTP (Amersham). Hybridization was conducted using 10% dextran sulfate, 40% formamide, 0.750 M $NaCl_2$ and 0.075 M Na citrate at 42°C (14). Stringency of the hybridization was set using washing conditions of 0.015 M NaCl, 0.0015 Na citrate and 52°C for EGF receptor probes.

RESULTS

Identification of human tumor cell lines with elevated EGF binding

In order to examine the involvement of growth factor receptor abnormalities associated with neoplastic transformation of human cells, we screened more than 50 human tumor cell lines representing most of the commonly occurring cancers for their ability to specifically bind radioactively labelled PDGF and EGF. Although none of the cell lines showed significant changes in their ability to specifically bind PDGF (data not shown), substantial variation was detected in the cell surface binding activity for EGF. We selected 5 tumor cell lines for further study (Table 1). These displayed specific EGF binding between 6 and 13 times the level found for M413 human embryo fibroblasts. These included BT20, HA698, A388, HA1781, and HA604. As shown in Table 1, the

Table 1. Specific EGF Binding Levels in Human Tumor Cells

Cell		EGF binding		
		Specific cpm bound 1000 cells	Specific cpm bound ∫g protein	Relative binding ∫g protein
M413	Normal fibroblast	10.9	7.3	1.0
A431	Epidermoid carcinoma	154.0	36.0	19.0
BT20	Mammary carcinoma	155.0	97.0	13.0
HA698	Gall bladder carcinoma	88.0	70.0	9.6
A388	Epidermoid carcinoma	80.0	70.0	9.6
HA1781	Glioblastoma	103.0	50.0	6.8
HA604	Gall bladder carcinoma	43.0	46.0	6.3

well characterized A431 epidermoid carcinoma cell line displayed specific binding 19 times that of M413 cells under the same assay conditions.

EGF receptor protein levels

In order to determine if the increased levels of specific EGF binding correlated with a higher concentration of EGF receptor protein, we undertook immunoprecipitation analysis using a monoclonal antibody specific for the EGF receptor. Equivalent numbers of cells were metabolically labelled with ^{35}S-methionine, the cell membrane lysates were precipitated with antibody, and were subjected to SDS polyacrylamide gel electrophoresis. Since we employed an excess of antibody in each reaction, the intensity of the observed signal gives an estimate of the quantity of EGF receptor present in the cell lysates. As shown in Figure 1, intense bands of 150-170 kd, the size of the EGF receptor, were specifically immunoprecipitated with each cell line examined. Each of the tumor cell lines analyzed demonstrated significantly more EGF receptor protein than that detected in the M413 embryo fibroblasts. These results indicated that the elevated level of EGF binding observed with these human tumor cell lines (Table 1) was likely to result from an increase in the quantity of EGF receptor protein available for binding.

Amplification of EGF receptor gene

To investigate the molecular mechanisms responsible for the increased levels of EGF receptor protein observed, we examined the structure of the EGF receptor gene in each cell line using Southern blot hybridization with a probe of cDNA (PE7) derived from the mRNA encoding the EGF receptor (7). This cDNA probe contains coding information corresponding to codons 120 to 919 within the region encoding mature EGF receptor consisting of 1186 codons. This orients

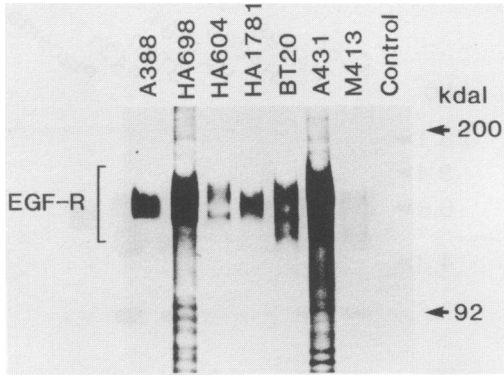

Fig. 1. Immunoprecipitation analysis of EGF receptor protein. Cell lines were metabolically labelled with S^{35}-methionine, lysed, equivalent membrane fractions prepared, equivalent amounts of incorporated radioactivity were immunoprecipitated with an excess of anti-EGF receptor antibody, separated by SDS polyacrylamide gel electrophoresis and detected by autoradiography. The lane marked "control" represents the immunoprecipitation reaction of an A431 membrane fraction using preimmune serum.

the cDNA probe within the mRNA as corresponding to a region beginning 0.6 kb from the 5' end of the mRNA and extending for 2.3 kb. Figure 2 shows the pattern of hybridization to the Eco RI digestion products of the EGF receptor gene of each cell line. In each case, 10 distinct bands were detected. Moreover each band was equivalent in molecular size to a corresponding band detected in DNA derived from human placenta. It should be noted that no abnormal restriction fragments are detected in the A431 cell line even though this cell line contains EGF receptor genes rearranged in the region homologous to the cDNA probe (7). This difficulty in detecting the EGF receptor gene rearrangement in A431 cells has been observed previously (5-8). However, three of the lines exhibited an increased hybridization signal intensity for each of the DNA fragments detected with the probe. These results implied that the BT20 and A388, in addition to the A431 cell line, may contain increased levels of the EGF receptor gene.

To provide a quantitative measure of the extent of amplification, we used a dot blot procedure in which successively smaller amounts of genomic DNA were applied to a filter and the EGF receptor gene detected using an excess of the EGF receptor cDNA probe. As shown in Figure 3, the amount of EGF receptor gene amplification in A431 cells can be estimated as about 8-fold, 4- to 8-fold in the BT20 cell and about 2-fold in the A388 cell. The other cell lines contained an apparently normal complement of EGF receptor genes.

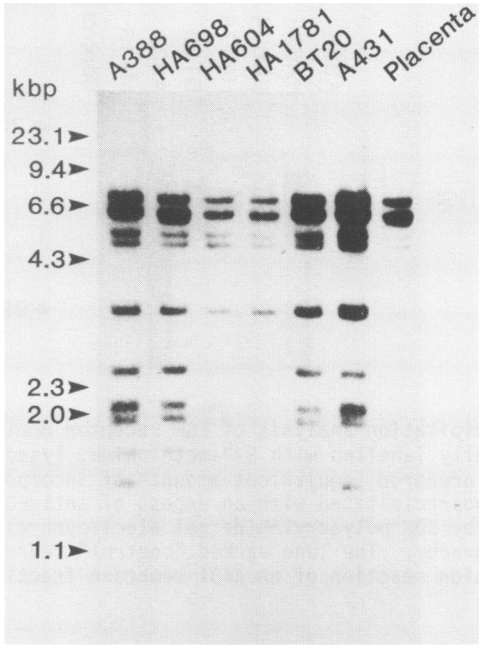

Fig. 2. Analysis of EGF receptor gene fragments. DNAs were digested (15 {g) with Eco RI separated by agarose gel electrophoresis transferred to nitrocellulose paper and detected by hybridization using radioactive clone EGF receptor cDNA as a probe.

As an internal control, hybridization using human c-mos as a probe indicated similar quantities of its gene in each of the same blots.

Lack of EGF receptor mRNA rearrangements

Extensive studies of the EGF receptor gene of the A431 cell have indicated that in addition to gene amplification, EGF gene rearrangement results in the production of an aberrant EGF receptor-related mRNA, of 2.7 kb, not present in mRNA of human placenta (5-8). An aberrant EGF receptor gene-related transcript has been reported in a primary human glioblastoma (9). To determine if any of the presently analyzed tumor cell lines which display elevated EGF receptor also contain such aberrant mRNA, we performed Northern blots using the EGF receptor cDNA as a probe. As shown in Figure 4, no aberrant EGF receptor transcripts could be detected in any of the lines. The faint band just below 28S in size observed in the lane containing A388 RNA is a non-specific effect of the presence of ribosomal RNA. Similar bands can be seen in long exposures with all RNA samples. While the transcript of the rearranged

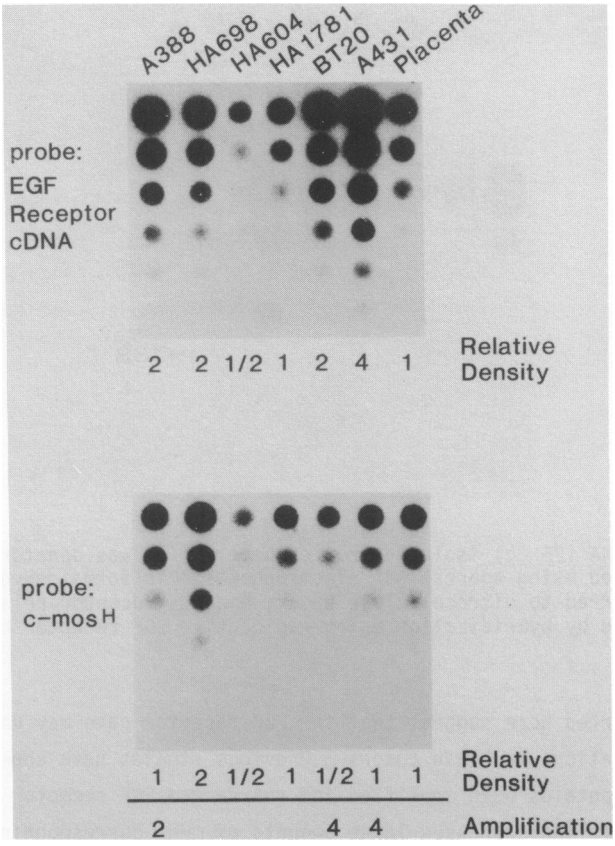

Fig. 3. Comparison of EGF receptor gene levels. DNA was denatured, neutralized and a 2-fold dilution series applied to nitrocellulose paper. The EGF receptor gene or human c-mos gene was detected by hybridization using radioactive EGF receptor cDNA or cloned human c-mos DNA as a probe. Relative densities of hybridization are indicated as a comparison to hybridization signals detected using placenta DNA.

EGF receptor gene of the A431 was readily detected, only the well characterized normal EGF receptor mRNAs of 10 kb and 6 kb were observed in any of the other tumor cell lines or in RNA of M413 embryo fibroblasts.

DISCUSSION

The characterization of cloned cDNA for the EGF receptor mRNA has stimulated a number of investigators to study the molecular alterations to the EGF receptor gene which may be associated with naturally occurring malignancies.

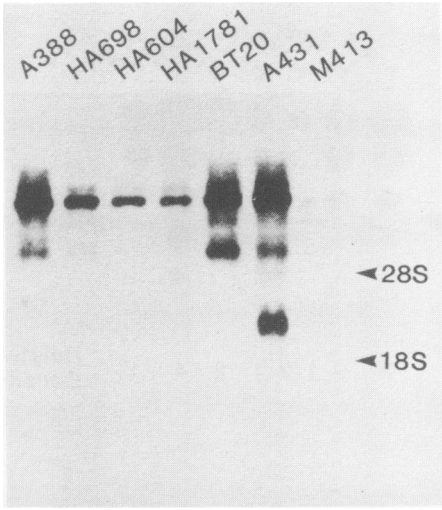

Fig. 4. Total RNA (25 {g) isolated from each cell line was denatured, separated using agarose gel electrophoresis in formaldehyde, transferred to nitrocellulose paper, and EGF receptor-related RNAs detected by hybridization using radioactive EGF receptor cDNA as a probe.

The results reported here suggest that the EGF receptor gene may undergo a variety of alterations in human tumors. Previous studies have shown that the cell line A431 contains both amplified and rearranged EGF receptor genes (5-8). This cell line expresses large amounts of mRNA corresponding in size to the normal EGF receptor mRNA as well as aberrant sized transcripts of the rearranged EGF receptor gene. Amplification and rearrangement of the EGF receptor gene has also been detected in primary human glioblastomas (9). Moreover, in such glioblastomas, EGF receptor gene rearrangement was associated with the expression of aberrant sized transcripts.

In the present studies, we report that the mammary carcinoma cell line, BT20, contains an EGF receptor gene amplification of a magnitude comparable to that observed in A431 cells but without rearrangements that cause the transcription of an aberrant mRNA as previously reported for A431 and certain human glioblastomas (5-9). In BT20 cells, amplification of this gene was accompanied by concomitant increase in the level of EGF receptor protein and EGF binding (68% of the A431 amount). We also detected a subtle amplification of the EGF receptor gene. An approximately 2-fold increase in gene copies observed in A388 cells may contribute to the striking increases in cell surface binding activity

displayed by these cell lines. However, gene amplification was found not to be a requirement for the marked increase in expression of EGF receptors (10). We observed three lines which displayed no evidence of increased EGF receptor gene copy number or rearrangement, but which nevertheless displayed 50% of the EGF binding sites of the A431 cell. These findings are in agreement with recent studies indicating that a number of human tumor cell lines express high levels of EGF receptors in the absence of detectable gene amplification (10). Taken together three distinct classes of molecular alterations affecting the EGF receptor gene can be identified: gene amplification accompanied by aberrant sized mRNAs, gene amplification without such altered RNAs, and undetectable amplification associated with markedly increased EGF receptor expression.

Alterations of the EGF receptor gene have now been found in human tumors from a variety of cellular origins. Amplification with rearrangement has now been identified in a human glioblastoma (9), and in cell lines derived from vulva (5-8) and squamous cell carcinoma (15, 16).

Gene amplification is often associated with phenotypic change. Gene amplification of the dihydrofolate reductase gene can confer a selective advantage to cells growing in the presence of methotrexate (17). In human malignancies, amplification of c-*myc* and n-*myc* genes appears to be correlated with the progression of lung carcinoma and neuroblastoma, respectively, to more malignant phenotypes (18, 19). Increased expression of normal *ras* genes can confer the transformed phenotype to NIH/3T3 cells. While the great majority of *ras* oncogenes of human tumors are activated by point mutations at one of two major "hot spots" in their coding sequences (20-22), certain tumors have also been found to contain amplified *ras* genes (23). Thus, accumulating evidence argues that amplification of known proto-oncogenes may be part of the selection for more malignant variants. If so, then EGF receptor gene amplification accompanied by increased expression of EGF receptors may confer a selective growth advantage in certain tumors independent of any other rearrangement affecting this gene. Our findings further indicate that other mechanisms, including at least one in which gene amplification is not required, can lead to markedly increased levels of EGF receptor expression. The detailed mechanisms associated with this phenotype selection remains to be elucidated.

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