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Advances in Brief

Frequent Expression of a Mutant Epidermal Growth Factor Receptor in Multiple Human Tumors[1]

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Abstract

The epidermal growth factor receptor has received much interest as a target for various antineoplastic agents, but a complication is that many normal tissues also express this receptor. We have previously identified in human glial tumors an 801-bp in-frame deletion within the epidermal growth factor receptor gene that created a novel epitope at the junction. By using Western blot assays with a mutant-specific antibody as a rapid and sensitive means for detecting this alteration in primary human tumors, it was found that 57% (26 of 46) of high-grade and 86% (6 of 7) of low-grade glial tumors, but not normal brain, express this protein. This altered receptor was also present in 66% (4 of 6) of pediatric gliomas and 86% (6 of 7) of medulloblastomas, 78% (21 of 27) of breast carcinomas, and 73% (24 of 32) of ovarian carcinomas. The fact that this receptor is frequently found in tumors but not in normal tissue makes it an attractive candidate for various antitumor strategies.

Introduction

The EGF[4] receptor has been implicated in the pathogenesis of multiple human tumors. High levels of the receptor have been found in 30–40% of breast carcinomas where expression is inversely correlated with the expression of the estrogen receptor and appears to confer a worse prognosis (1). In astrocytic neoplasms, amplification of the *EGF* receptor gene is preferentially associated with high-grade tumors (grades III and IV), where it is present in ~40% of glioblastoma multiforme (2). Overexpression of the receptor has also been noted frequently in breast, bladder, and ovarian tumors, and in various squamous carcinomas (1). Transfection studies on rodent fibroblasts have shown that overexpression of the EGF receptor can result in the acquisition of the transformed phenotype (3). For these reasons this receptor has been thought to be an extremely attractive target for rationally designed therapeutics. It has been shown that monoclonal antibodies conjugated to ^{131}I can achieve tumor regression in mice bearing tumors that overexpress the receptor (4). A Phase II clinical trial is currently underway to evaluate the efficacy of monoclonal antibodies targeting this receptor in patients with glioblastoma (5).

Because normal EGF receptors may also be targeted by such agents, it would be desirable to define alterations within the receptor that are tumor specific. Several reports have documented spontaneous rearrangements within the *EGF* receptor gene that arose in primary human glioblastoma tumors (6–8). These alterations were always in-frame deletions that preserved the reading frame of the receptor message. The most common of these rearrangements was the EGFRvIII, which involves a deletion between nucleotides 275–1075 in the normal EGF receptor cDNA sequence (6, 8, 9). By Southern blot analysis, 17% of glioblastoma tumors have a deletion that gives rise to this variant receptor. A synthetic peptide that spans the junction can be used to elicit an antibody that specifically recognizes the EGFRvIII but not the normal EGF receptor (9). We have subsequently used this antibody to show that this receptor is present in 16% of non-small cell carcinoma of the lung tumors (10). In this study, we have used Western blots with this antibody to detect this altered receptor in several types of human tumors. Using this method, we have found that the EGFRvIII is more prevalent in adult glial tumors than was previously thought and was also frequently detected in primary breast, ovarian, pediatric glioma, and medulloblastoma tumors.

Materials and Methods

Tumors and Cells. All diagnoses were confirmed by histological analysis and specimens were trimmed to remove any normal tissue. RNA was obtained from tumor 256, a human glioblastoma maintained as a xenograft in athymic mice (11). HC2 20d2/c is derived from NIH-3T3 cells transfected with a cDNA encoding the EGFRvIII.[5] This cell line expresses a level of the EGFRvIII equivalent to that found in glioblastoma tumors with amplification of the EGFRvIII rearrangement (12). The normal human ovarian protein was from Clontech (Palo Alto, CA).

Antibody against the EGFRvIII. Antibody against the EGFRvIII was prepared as described previously (9). Briefly, the peptide used for immunization in single-letter code was LEEKKGNYVVTDHC (Immuno-Dynamics, Inc., La Jolla, CA) where the underlined glycine is the novel amino acid created by the fusion of the two normally distant sequences, and the terminal cysteine was added for purposes of conjugation. This peptide was conjugated to keyhole limpet hemocyanin and used to immunize New Zealand White rabbits. Antibody was purified by affinity chromatography using the same peptide linked to CNBr-activated Sepharose 4B (Sigma Chemical Co., St. Louis, MO).

Western Blot Analysis of Tumor Lysates. Tumors were homogenized in PBS/TDS buffer [10 mM Na_2HPO_4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.2% sodium azide, and 0.004% sodium fluoride (pH 7.25)] containing 1 mM sodium orthovanadate. Protein concentrations were determined by the BioRad DC assay (BioRad, Richmond, CA). Lysates (50 or 150 μg as specified) were electrophoresed on 6% (0.75 mm) or 4–20% (1.0 mm) SDS-PAGE gels. Gels were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) by using a standard protocol. Membranes were blocked in Blotto/TTBS [100 mM Tris (pH 7.5) 150 mM NaCl, and 0.1% Tween 20 containing 5% nonfat dry milk]. The blots were then incubated with 1 μg/ml of anti-EGFRvIII or monoclonal antibody against an intracellular epitope of the human EGF receptor (Zymed, San Francisco, CA)

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[4] The abbreviations used are: EGF, epidermal growth factor; EGFRvIII, type III mutant EGF receptor; RT-PCR, reverse transcription-PCR.

[5] D. K. Moscatello, R. B. Montgomery, P. Sundareshan, H. McDanel, M. Y. Wong, and A. J. Wong, submitted for publication.

in Blotto/TTBS for 2 h at room temperature, washed four times in TTBS, and then incubated with 0.23 μCi/ml of ^{125}I antirabbit IgG or antimouse IgG antibody (Amersham, Arlington Heights, IL) for 1 h, washed four times, dried and exposed to film.

Peptide Competition. Western blots of breast tumor lysates were exposed to anti-EGFRvIII antibody at 1 μg/ml in the presence or absence of 5 μg/ml of free immunizing peptide and subsequently processed as described above.

RT-PCR-based Analysis of Transcripts. RNA was extracted from tumors by using the RNAzol method (Tel-Test, Inc.). First-strand cDNA synthesis and subsequent PCR was performed as described previously (8). PCR samples were then electrophoresed in 2% agarose gels, transferred to Zeta-Probe GT membranes (BioRad, Hercules, CA), and the blots were probed with an EGF-receptor cDNA probe spanning the EGFRvIII fusion junction.

Results

Detection of EGFRvIII by Western Blot Analysis

Because Western blot analysis possesses several advantages it was chosen as the primary means for assessing the presence of the EGFRvIII. The only sample preparation required is tissue homogenization, unlike RNA isolation which requires several subsequent steps, and the EGFRvIII protein appears less labile than RNA. Western blots permit confirmation that the immunoreactive molecule is the proper size and also allow for quantitative comparisons. To demonstrate that the anti-EGFRvIII antibody specifically recognized this altered receptor, we performed Western blots with this antibody on lysates from the HC2 20d2/c cell line, which expresses ~2×10^6 mutant receptors per cell (Fig. 1*A*; Ref. 12). For comparison, lysates from A431 human epidermoid carcinoma cells, which express ~1×10^6 normal EGF receptors/cell, and normal human fibroblasts, which contain ~1×10^4 receptors/cell, were also analyzed. The anti-EGFRvIII antibody intensely labeled a series of bands from M_r ~100,000 to 140,000 in the HC2 20d2/c cell line. The predicted size for the EGFRvIII is M_r 104,000 but this receptor is probably glycosylated *in vivo* much like the normal EGF receptor. This antibody only faintly recognized the M_r 170,000 normal EGF receptor band in A431 cells and did not detect this band in normal human fibroblasts (Fig. 1*A*). In contrast, Western blots performed with an antibody that recognizes a carboxyl-terminal epitope of the EGF receptor (Fig. 1*B*) showed approximately the same amount of reactivity in A431 and HC2 20d2/c cells and was able to detect the receptor in the normal human fibroblasts. This demonstrated that the anti-EGFRvIII antibody almost exclusively recognized this altered receptor.

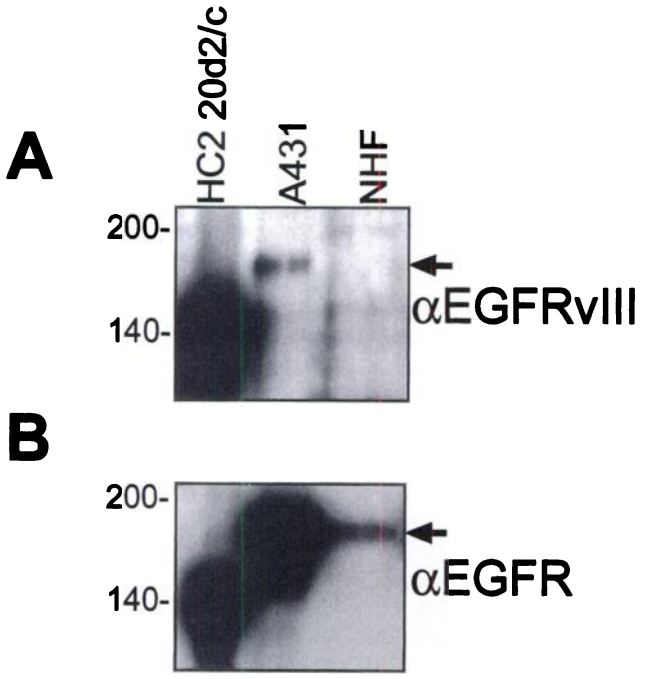

Fig. 1. Affinity-purified anti-EGFRvIII antibody is specific for the EGFRvIII rearrangement. Lysates of HC2 20d2/c (50 μg), A431 cells (150 μg), and normal human fibroblasts (*NHF*, 100 μg) were separated on a 6% SDS-PAGE gel and transferred to nitrocellulose, and duplicate blots were probed with either the affinity-purified anti-EGFRvIII antibody (αEGFRvIII) or the monoclonal antihuman EGF receptor antibody (αEGFR). *Arrows*, position of the normal EGF receptor. No reaction of the αEGFRvIII antibody is detectable with the amount of EGFR present in normal human fibroblasts, and only weak cross-reaction is seen with the A431 cell line, which overexpresses the normal EGF receptor.

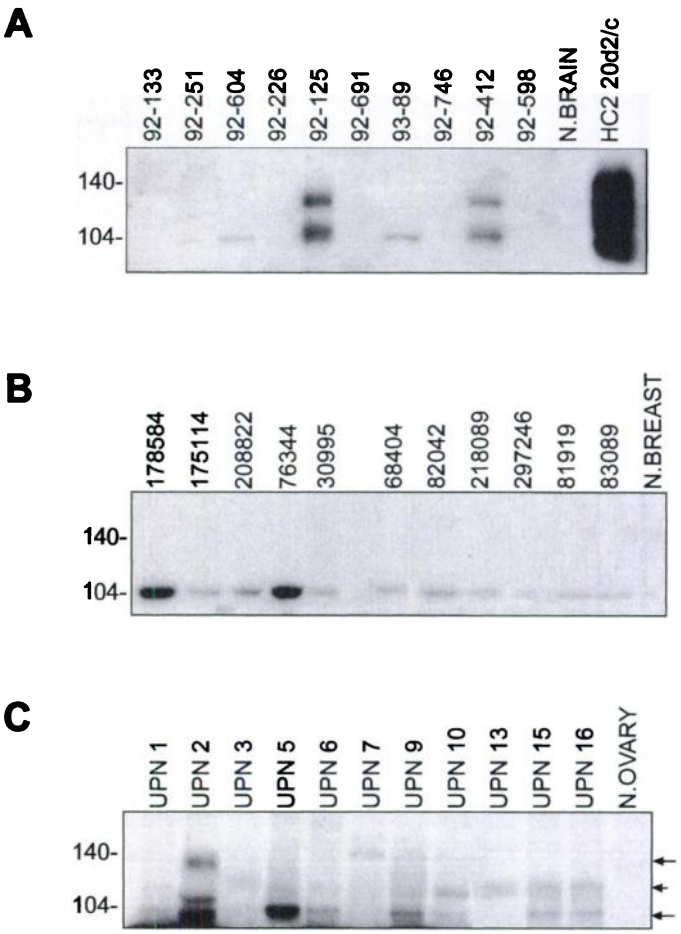

Fig. 2. Expression of EGFRvIII in brain, breast, and ovarian cancers. Indicated amounts of homogenate protein from tumor specimens were separated on 6% (astrocytoma and breast) or 4–20% gradient (ovarian) SDS-PAGE gels, transferred to nitrocellulose, and probed with anti-EGFRvIII antibody. *A*, grade III/IV astrocytomas (50 μg). Note the presence of both M_r ~140,000 and M_r ~100,000 bands in two of the tumors and lesser amounts of only the M_r ~100,000 bands in several of the others. *B*, breast carcinoma homogenates (100 μg). Note the presence of varying amounts of a M_r ~100,000 band in many of the samples. *C*, ovarian carcinoma homogenates (40 μg). Note the presence of the M_r 140,000 bands in some of the tumors, as well as both M_r ~100,000 and M_r 125,000 bands in many of the tumors (*arrows*). *N*, normal tissue from human brain, breast, or ovary.

Identification of the EGFRvIII in Multiple Human Tumors

Brain Tumors. We found expression of the EGFRvIII at extremely high levels in ~17% of grades III and IV tumors, which correlates with the incidence of gene amplification for this mutant receptor (6, 8, 9). Most tumors, although, expressed a low to moderate level of a M_r ~100,000 band; the M_r 140,000 band was primarily found in those tumors expressing a high level of the EGFRvIII (Fig. 2*A*). The presence of nonglycosylated EGFRvIII at lower levels of

expression may reflect a predominantly intracellular localization of this particular mutant (13). Overall, we detected an EGFRvIII-specific band in 56% (35 of 62) of these tumors. No reactivity was found in a normal brain specimen even when up to 150 μg of sample was loaded (Fig. 2A; data not shown). Although amplification of the *EGF* receptor gene is commonly found in adult grade III and IV tumors, it is infrequently found in grade II or pediatric gliomas. However, the EGFRvIII was found in 86% (6 of 7) of grade II and 66% (4 of 6) pediatric gliomas (Table 1), indicating that the presence of this protein could be found in these less aggressive tumors and was not associated with the factors that led to gene amplification. We also found the EGFRvIII in 86% (6 of 7) of pediatric medulloblastomas.

Breast Tumors. We examined 27 breast carcinomas (which were all infiltrating ductal carcinoma) for the presence of the EGFRvIII. By Western blotting, the mutant receptor was found in 21 of 27 (77.8%) breast carcinomas (Fig. 2*B*). This is similar to the incidence reported in a smaller number of tumors using RT-PCR, but substantially higher than what was detected by immunohistochemistry (27%; Ref. 12) indicating the level of sensitivity of Western blot analysis. As with normal brain, this band was not detected in up to 150 μg of normal breast tissue. To confirm that the M_r 100,000 band seen in Western blots was specific to the EGFRvIII, Western blots were performed in the presence and absence of the peptide that corresponded to the amino acid sequence at the fusion junction of the mutant receptor. As shown in Fig. 3*A*, the peptide abolished the binding of the anti-EGFRvIII antibody to the M_r 100,000 band in the two breast tumor samples examined. Additional confirmation was obtained by analyzing specimens for the presence of EGFRvIII-specific transcripts. RNA was isolated from frozen sections of two breast tumors that had low levels of EGFRvIII expression by Western blot analysis. RT-PCR was performed on the RNA by using oligonucleotides encoding amino acid sequences flanking the fusion junction. A 263-bp band corresponding to the mutant receptor could be detected in these specimens and in the positive control sample (Fig. 3*B*).

Ovarian Tumors. We found expression of the EGFRvIII in 24 of 32 (75%) ovarian carcinomas tested but not in normal ovary. Histological information was available on 24 tumors that showed that EGFRvIII was present at a high percentage in all grades (Table 1). Seven tumors had detectable levels of the M_r ~140,000 band (20.6%), whereas 18 expressed the M_r ~100,000 band. Tumors with the M_r 140,000 band also had the M_r 100,000 band except for one tumor that solely expressed the M_r 140,000 form (Fig. 2*C*, *UPN7*). Diffuse bands of M_r ~110,000–125,000 were visible in 12 of the tumors that were positive for EGFRvIII expression; these bands have been observed previously only in glioblastomas expressing very high levels of the EGFRvIII. The types of bands expressed did not appear to be associated with tumor grade. Expression of the EGFRvIII was also detected in five of eight ovarian carcinoma cell lines (data not shown).

Table 1 *Expression of EGFRvIII in human tumors*

Tumor	EGFRvIII expression in %
Astrocytomas	56 (35/62)
Grade II	86 (6/7)
Grade III/IV	53 (29/55)
Medulloblastomas	86 (6/7)
Pediatric gliomas	66 (4/6)
Breast carcinomas (infiltrating ductal)	78 (21/27)
Ovarian carcinomas	75 (24/32)
Grade I	66 (2/3)[a]
Grade II	78 (7/9)
Grade III	92 (11/12)

[a] Histopathologic grade was not available for all tumors.

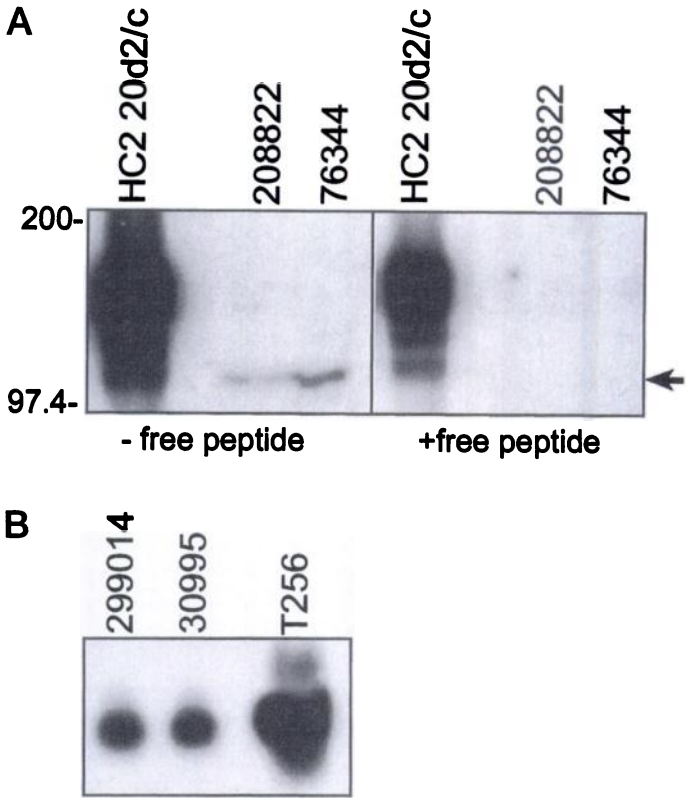

Fig. 3. The M_r 100,000 band recognized in breast tumor lysates is derived from EGFRvIII. *A*, competition with peptide corresponding to the EGFRvIII fusion junction abolishes antibody binding to the M_r 100,000 band. Binding to the EGFRvIII overexpressed in HC2 20d2/c was also substantially competed, but the blots were overexposed to visualize the M_r 100,000 bands in the breast tumors. *B*, detection of EGFRvIII mRNA in breast carcinomas by RT-PCR. RT-PCR using oligonucleotides encoding amino acid sequences flanking the fusion junction was performed on RNA isolated from frozen sections of two breast tumors that had low levels of EGFRvIII expression by Western blot. The autoradiogram shows the 263-bp band corresponding to the mutant receptor in these specimens and the positive control sample (tumor 256).

Discussion

In this paper we report the detection of the EGFRvIII in a high proportion of epithelially derived tumors. Interestingly, rearrangements within the *EGF* receptor gene have been detected previously by Southern blot analysis in squamous cell carcinomas (14), ovarian carcinomas (15), and lung carcinomas (16). Several of the reported gene rearrangements resemble those that are known to give rise to the EGFRvIII protein in glial tumors; therefore, one possible origin for this altered receptor is via gene rearrangement (8). Because the EGFRvIII is a result of the joining of exon 1 to exon 8 of the *EGF* receptor gene, it could arise through alternative splicing. This appears to be the case for the breast carcinomas because Southern blot analysis of these tumors did not reveal any rearrangements (data not shown). The presence of high levels in ovarian cancers raises the possibility that there may be amplification of an altered receptor gene in these tumors.

Several features of the EGFRvIII make it an excellent target for biologically based therapies. Down-regulation of this receptor may remove an important growth stimulus to tumor cells. We have derived an NIH-3T3 cell line, HC2 20d2/c, that expresses levels of the EGFRvIII comparable to that found in glioblastoma multiforme tumors. In HC2 20d2/c cells, the mutant receptor is constitutively active in the absence of ligand, resulting in a transformed morphology, enhanced growth, and tumorigenicity in athymic mice.[5] Other investigators have also noted that the EGFRvIII is constitutively active (17)

and that expression of this receptor can enhance the tumorigenicity of the U87 glioma cell line (18).

Several therapeutic approaches now exist that can be developed to exploit the presence of this mutant receptor. Recent studies have shown the feasibility of at least two such avenues. Because the EGFRvIII is expressed on the cell surface in some tumors (9), it would be accessible to antibodies or other antireceptor reagents. Immunization of mice with HC2 20d2/c cells has resulted in the successful production of several high-affinity monoclonal antibodies against this receptor that can be internalized (19). Treatment of animals with *Pseudomonas* exotoxin-conjugated monoclonal antibodies can reduce the size of tumors expressing the EGFRvIII (20). Alternatively, because the presentation of peptides by MHC molecules does not depend on cellular localization, peptide vaccination may present another route. We have found that mice given injections of a peptide vaccine corresponding to the novel junction of EGFRvIII can prevent or induce the regression of existing tumors in immunocompetent mice given injections of HC2 20d2/c cells. The immune response generated was mainly mediated by $CD8^+$ T lymphocytes.[6]

Including[1] the data from this report, we have now demonstrated that the EGFRvIII is present in brain, breast, lung, and ovarian tumors. This variant is tumor specific because in previous work we analyzed a large number of adult tissues that did not reveal the presence of the EGFRvIII (9, 10). Considering the incidence of all these tumors and the frequency of this receptor alteration, the number of patients that could be potentially treated is >150,000/year. These facts coupled with the unique properties of the receptor make a compelling case for therapeutic strategies directed against this receptor.

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