Expression of Mutated Epidermal Growth Factor Receptor by Non-Small Cell Lung Carcinomas

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Abstract

The development of novel immunotherapy strategies for non-small cell lung cancer (NSCLC) will be facilitated by the identification of tumor-specific targets. Although the epidermal growth factor receptor (EGFR) is overexpressed in many cases of NSCLC, its wide distribution in normal tissue may limit its suitability as an immunotherapeutic target. However, mutations within the EGFR that are unique to malignancies may provide specific targets for immunotherapeutic intervention. For example, one mutant form, the type III deletion mutant of the EGFR, that has been identified in glioblastomas contains a novel peptide sequence in its extracellular domain which is detectable by anti-peptide antisera. In this study, the prevalence of this type of mutation of the EGFR in NSCLC was determined. Thirty-two frozen sections of primary NSCLC were examined by immunocytochemistry to determine the presence of native and mutated EGFR. Native EGFR was overexpressed in 12 of the 32 sections and a diffuse cellular distribution of the EGFR type III deletion mutation was identified in five (16%) of the specimens (2 of 13 squamous, 2 of 2 mixed, 0 of 10 adenocarcinoma, and 1 of 7 undifferentiated). This mutated EGFR was not detected in sections of normal breast, lung, skin, ovary, colon, kidney, endometrium, and placenta. The type III EGFR deletion mutant, expressed in some cases of NSCLC, may be a molecularly defined, tumor-specific antigen in lung cancer.

Introduction

NSCLC is the most frequent cause of cancer death in the United States. Cure rates are directly related to clinical stage (70% for Stage I, decreasing to less than 5% for Stage IIIB) (1). Therapy using various permutations of surgery, radiotherapy, and chemotherapy has had limited effects on cure rates and median survival in advanced stages of NSCLC (1). Clearly, novel therapeutic targets and agents are needed for the management of this disease. One such approach involves immunotherapy directed against antigens which are either selectively expressed or over expressed in malignant cells. A number of potential targets have been identified; the potential suitability of the EGFR as a target has been the subject of intensive study.

Overexpression of EGFR has been associated with a number of neoplasms, including breast carcinoma (2, 3), adenocarcinoma and squamous cell carcinoma of the lung (3–7), large cell carcinoma of the lung (3, 5, 6), gliomas (8), and a variety of bladder (9, 10) and gynecological tumors (11). In both bladder and breast cancer, a poor prognosis has been correlated with high expression of EGFR (2, 3, 9, 10). Due to its high expression by a number of neoplasms, the EGFR has been utilized as a tumor-associated target for tumor detection and therapy. In imaging studies performed in patients with squamous cell carcinoma of the lung, over 94% of primary lesions and sites of presumed metastases were detected when doses of 40 mg or more monoclonal antibody were utilized (12, 13). In mice bearing a lethal inoculum of tumor cells expressing EGFR, treatment with transforming growth factor-α-Pseudomonas exotoxin 40 fusion proteins more than doubled median survival. The fusion protein did not significantly prolong the survival of mice inoculated with EGFR-negative tumor cells (14, 15).

Amplification of the EGFR gene has frequently been associated with EGFR overexpression in gliomas (8, 16, 17) and has been reported in squamous cell carcinomas (including those of the lung) (18–20), breast carcinomas (21), and bladder tumors (9). Rearranged or truncated forms of EGFR have been identified and are often associated with gene amplification in gliomas (22–26). To date, three truncated forms of EGFR have been reported. The type I deletion mutant lacks the majority of the extracellular domain and is unable to bind epidermal growth factor (26). The type II deletion mutant contains an in-frame deletion of 83 amino acids (520–603) in domain IV of its extracellular domain and is capable of binding epidermal growth factor and transforming growth factor α (23). The type III deletion mutant, reported to occur in 17% of the glioblastomas screened by Humphrey et al. (22), appears to be the most prevalent. This mutation generates a fusion junction sequence which is reported to be unique to malignancies. This sequence results from an in-frame deletion of 267 amino acids (from amino acids 29–296) spanning the first and second extracellular domains of the receptor (22, 26, 27). A synthetic peptide spanning the deletion junction (H-Leu-Glu-Glu-Lys-Lys-Gly-Asp-Tyr-Val-Val-Thr-Asp-His-Oh) has been used to raise antisera specific for this mutant protein. To determine if this mutated growth factor receptor merits consideration as a tumor-specific target in NSCLC, 32 sections of primary NSCLC were studied for the presence of the type III EGFR deletion mutation.

Materials and Methods

Antibodies. Rabbit and goat antiserum specific for the EGFR type III deletion sequence were prepared as described (22). Briefly, a 14-amino acid peptide spanning the predicted amino acid sequence of the fusion junction was synthesized, purified, and chemically conjugated to keyhole limpet hemocyanin. Animals were immunized with a 1:1 emulsion of the conjugate in PBS and complete Freund's adjuvant. Boosts were performed on day 33 with conjugate in a 1:1 emulsion with incomplete Freund's adjuvant. The animals were bled and the anti-peptide antibody was purified from the antisera on a peptide-Sepharose affinity column.

Immunohistochemistry. Frozen sections of NSCLC and normal tissues were obtained from the Fox Chase Cancer Center Tumor Bank. The human glioblastoma multiforme xenograft (D-270 MG-X), expressing the type III EGFR deletion mutation, was utilized as a positive control. The sections were stained with hematoxylin-eosin to evaluate histology. Five-μm sections were fixed for 20 min in cold acetone and air-dried at room temperature. The...
sections were then incubated in Tissue Conditioner Reagent (Biomeda Immunohistochemical Staining Kit, Foster City, CA) for 10 min at room temperature. A blocking reagent (normal rabbit or goat serum) was then added and the sections were incubated at room temperature for 30 min. The blocking reagent was blotted and the samples were rinsed with 0.01 M PBS, pH 7.2. The sections were then incubated overnight at 4°C with 5 μg/ml of primary antisera reactive with the type III deletion junction sequence, normal rabbit/goat serum, or 3 μg/ml of affinity purified rabbit antibody to native EGFR (Ab-4; Oncogene Science, Inc., Manhasset, NY). After the slides were washed with PBS, 100 μl of a 1:1 dilution of biotinylated goat anti-rabbit IgG (BioGenex Laboratories, San Ramon, CA) or rabbit anti-goat IgG (Bioment) as appropriate were applied and allowed to incubate at room temperature for 45 min. The sections were then washed twice with PBS and incubated with 100 μl of streptavidin alkaline phosphatase for 45 min at room temperature. They again were washed with PBS and incubated with 100 μl of Fast Red Chromogen (naphthol phosphate) (BioGenex Laboratories). The slides were washed in PBS and counterstained with Mayer’s hematoxylin (Sigma Diagnostics, St. Louis, MO) for 5 min. The validity of immunohistochemical detection of cells expressing the type III EGFR deletion mutation in gliomas, using these antisera, previously has been confirmed by Western blot analysis (22).

Karyotypic Analysis. In one case in which a fresh specimen was available, tissue was disaggregated by mechanical means (28). Actively growing cells were arrested in metaphase by exposure to 0.03 μg/ml colcemid (GIBCO Laboratories) for 16-20 h. Cells were then treated with a hypotonic solution of 0.075 M KC1 for 20 min at 37°C. They were fixed in a 3:1 solution of methanol:glacial acetic acid. Chromosomes were analyzed using a G-bandning technique (29). Chromosome identification and karyotypic designation were performed in accordance with the ISCN (30).

Results

Immunohistochemistry. Twelve of the 32 sections (38%) of NSCLC were stained by affinity purified rabbit antibody to native EGFR (Ab-4) by the immunoperoxidase method (Table 1). Additionally, 5 of the 12 (16% overall) specimens with high levels of native EGFR also diffusely expressed the type III deletion mutant. Expression of this deletion mutant was limited to malignant squamous epithelial cells and was not observed in cells with adenocarcinoma differentiation. In the two positive cases of adenosquamous carcinoma, both native EGFR and the deletion mutant were observed only in the squamous cell component (Fig. 1). In the positive specimens, all of the squamous cells stained positive for the deletion mutant. All cells which were found to express the deletion mutant also reacted with the rabbit antisem to native EGFR. The type III deletion mutant was found to be predominantly associated with the cellular membrane, although in some cases cytoplasmic staining was observed. Normal tissue samples were screened for the presence of both native EGFR and the deletion mutant. Native EGFR was found to be highly expressed on skin (2) and endometrium (2), diffusely expressed on scattered cells in the lungs (2) and the colon (3), and not observed on breast (3), ovary (3), kidney (2), and placenta (5). Antiserum directed against the type III deletion mutant did not bind to any of the above normal tissues.

Table 1  Immunohistochemical detection of native EGFR and EGFR type III deletion mutant expressed in non-small cell lung carcinoma

<table>
<thead>
<tr>
<th>Histology</th>
<th>Expression of EGFR</th>
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<tr>
<td>Squamous</td>
<td>13</td>
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<td>Adenosquamous</td>
<td>2</td>
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<td>Adenocarcinoma</td>
<td>10</td>
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<tr>
<td>Undifferentiated</td>
<td>7</td>
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<tr>
<td>Total</td>
<td>32</td>
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* Number of samples.
* Percentage of samples positive for mutant.
* Positive staining only observed on squamous component.

Karyotypic Analysis. One sample of squamous cell carcinoma, positive for expression of the type III deletion mutant, was available for karyotypic analysis. This specimen was found to be near triploid. Most cells examined had four copies of chromosome 7 plus two copies of a translocation derivative, der(2)(q27;7), containing an extra portion of most of the short arm of chromosome 7 (Fig. 2). The derivative included the region containing the EGFR gene, presumably resulting in six copies of the gene/cell.

Discussion

This study provides a preliminary assessment of the prevalence of EGFR type III deletion mutant expression in NSCLC. The mutant was present in 16% of all NSCLC specimens we tested and in 42% of the cases in which overexpression of the EGFR was detected. The prevalence of the EGFR type III deletion mutant in NSCLC may actually be greater than reported here, because immunocytochemistry techniques are dependent upon both the sensitivity of the antibodies and detection methods which are used. For example, in this assay, we observed native EGFR expression in 38% of the NSCLC samples examined. However, EGFR expression in NSCLC, as detected by immunocytochemistry, has been observed in 52-83% of specimens studied using other reagents (9, 31).

Expression of the deletion mutant was observed only in malignant squamous epithelial cells. In the cases of adenosquamous carcinoma studied, only the squamous component was positively stained. Normal breast, lung, skin, ovary, colon, and endometrium failed to react with antisem against the deletion mutant fusion junction. The staining pattern of the positive cells revealed that the predominant expression of the EGFR type III deletion mutant was membrane associated. While cytoplasmic staining was observed in some cases, nuclear staining was never observed. Expression of the type III deletion mutant was confirmed by Western blot analysis in the two cases where frozen tissue blocks were available (results not shown).

Cytogenetic analysis of one squamous cell carcinoma revealed polysomy 7 and extra copies of most of 7p; the latter were contained within two identical translocation derivatives, der(2)(q27;7) (Fig. 2). The breakpoint on chromosome 7 in the der(2)(q27) is at 7p11.2-p12, which is near the location of the EGFR gene, but we do not know if this structural rearrangement has disrupted the EGFR locus. In a previous study, we observed polysomy for all or part of the short arm of chromosome 7 in 20 of 30 (67%) NSCLC specimens (32). The shortest region of overlap of partial gains of 7p was at region 7p11→p13, where the EGFR gene is located (33). While an increase in the number of copies of EGFR might be expected to manifest itself in elevated expression of this gene, the correlation between polysomy of 7p and increased EGFR levels in NSCLC will require further study.

The type III deletion mutant appears to be a promising target for therapy. Its fusion junction sequence is immunogenic. It is located near the amino-terminus of the extracellular domain, rendering it easily accessible to monoclonal antibodies. The detection of the EGFR type III deletion mutant by immunohistochemistry correlates extremely well with Western blot analysis and molecular detection of the deletion in tumor samples (22). Furthermore, antiserum directed against this sequence on EGFR type III mutant-expressing glioma cells (D-270 MG-X) has been demonstrated to be completely internalized within 60 min (22), making this type III EGFR mutant potentially useful as a target for immunotoxin therapy.

The frequency of expression of the EGFR type III deletion mutant in NSCLC (16%) is very similar to that reported in glioblastomas (17%) (22) and suggests that this deletion mutant may occur in a spectrum of EGFR-overexpressing malignancies. If this prevalence is verified by the study of more NSCLC samples, as many as 20,000 individuals annually afflicted with NSCLC in the United States will
express this deletion mutant and may be candidates for treatments which exploit the presence of this antigen. Mutations of the EGFR would not be likely to occur in normal tissues and have not been detected in the limited panel which has been examined thus far. Thus, mutated growth factor receptors may be molecularly defined tumor-specific antigens. If this is indeed the case, the EGFR type III deletion mutant may provide a unique and powerful target for a number of forms of cancer detection and treatment, since the lack of antigenic targets on normal tissue should reduce the uptake of reagents and resultant toxicity to nontargeted tissues.

These studies show that immunogenic mutations of the EGFR can be detected in at least two types of malignancies. Additional malignancies may be found to express the type III deletion mutation. Other mutations in the EGFR extracellular domain may be identified by systematically analyzing NSCLC and other tumor samples in which gene amplification of the EGFR is noted. Finally, it is possible that other growth factor receptors may undergo similar patterns of deletion mutations in the setting of malignant transformation, providing additional examples of molecularly defined, tumor-specific antigens which can be exploited as new therapeutic targets.

References

Fig. 1. Adenosquamous carcinoma of the lung. A, squamous component of the tumor. Tissue was stained as described in "Methods and Materials" using a biotinylated goat anti-rabbit IgG system following incubation with primary antibody to the type III deletion mutant. B, adenocarcinoma component of the same tumor. Sections were treated as described above. Note that this component shows no reactivity for the type III deletion mutant antibody. Frozen sections; Fast Red with hematoxylin counterstain (X 500).

Fig. 2. Chromosomes 2 and 7 from two different metaphase spreads (A and B) from a squamous cell lung carcinoma. Each metaphase has 3 or 4 copies of normal chromosome 7 and two copies of a translocation derivative chromosome 2 containing most of the short arm of chromosome 7 translocated to the distal end of chromosome 2 (arrows).


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