Detection of the Proto-oncogene eIF4E in Larynx and Hypopharynx Cancers

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**Background:** The proto-oncogene eIF4E has been found to be elevated in head and neck squamous cell carcinomas. In an earlier prospective study overexpression of eIF4E, detected by Western blot analysis, in histologically normal surgical margins correlated with an increased local-regional recurrence rate during a 1-year follow-up.

**Objective:** To test the reverse hypothesis that absence of overexpression of eIF4E in the surgical margins is a predictor for long-term survival in patients with squamous cell carcinoma of the head and neck.

**Design:** A retrospective analysis was performed on 31 patients who underwent surgery for squamous cell carcinoma of the larynx or hypopharynx. Immunohistochemical analysis was used to detect eIF4E on paraffin-embedded sections of the tumor and the histologically negative surgical margins.

**Results:** All 31 patients overexpressed eIF4E in the tumors. Thirteen patients had no detectable level of eIF4E in the margins, and only 1 had a local-regional recurrence. The average disease-free interval in this group of patients was 82.08 months. The remaining 18 patients all overexpressed eIF4E in the surgical margins (eIF4E score range, 5-80). Twelve (67%) of these patients developed a recurrence; the average disease-free interval was 31.95 months. Cox regression analysis showed that eIF4E in the margin (P = .01), nodes (P = .06), site (P = .02), and age (P = .02) had significant effects on the disease-free interval. The Kaplan-Meier survival curves were significantly different for eIF4E-positive and eIF4E-negative margins (P = .002).

**Conclusions:** eIF4E in the surgical margins is an independent prognostic factor and its absence in surgical margins may predict long-term survival. Detecting eIF4E in the margins may improve survival by determining which patients would benefit from further resection or adjuvant therapy.


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**The translation initiation factor eIF4E binds to the m7GpppX cap of messenger RNA (mRNA) in the first step of mRNA recruitment, and unwinds the secondary structure at the 5’ untranslated region (5’UTR) of mRNA as part of the eIF4F complex.**

The latter function is critical during scanning for exposing and locating the translation start site. eIF4E has recently been identified as a powerful oncogene when overexpressed in model cell lines. The oncogenic function of eIF4E is mediated in part by a specific increase in synthesis of certain growth factors, since conditioned medium from cells transformed with eIF4E is strongly mitogenic. A large translational increase of 2 powerful cytokines and angiogenic inducers: fibroblast growth factor 2 and vascular permeability factor was subsequently confirmed. Also, a reduction of eIF4E with antisense RNA in MDA-435 cells suppresses the tumorigenic and angiogenic properties, consistent with loss of fibroblast growth factor 2 synthesis.

We recently reported in a prospective study that eIF4E protein detected by Western blot analysis was elevated in all head and neck squamous cell carcinomas (HNSCC) and not in benign lesions of the head and neck. Its overexpression in histologically normal surgical margins correlated with a higher rate of local recurrence. Kaplan-Meier survival curves for eIF4E-positive and eIF4E-negative margins were significantly different by the log-rank test (P = .02); however, the average length of follow-up was only 7.8 months for the patients with eIF4E-positive margins and 13.6 months for the patients with eIF4E-negative margins. We therefore performed a retrospective study to determine whether the presence or absence of eIF4E in the surgical margins of patients...
PATIENTS, MATERIALS, AND METHODS

STUDY POPULATION

Thirty-one previously untreated patients who underwent surgery for a HNSCC of the larynx or hypopharynx at Louisiana State University Medical Center and the Veterans Hospital in Shreveport between 1980 and 1990 were studied. If any margins on the final pathology report were positive for cancer the patient was excluded from the study. A total of 83 “histologically tumor-free” surgical margins were analyzed. Medical charts were reviewed for the following factors: age, race, tumor size, node status, primary tumor site, histological grade of the tumor, postoperative radiation therapy, disease-free interval, and recurrence status.

SAMPLES

Paraffin-embedded tissue blocks from the primary tumor and all surgical margins were obtained. One slide from each block was stained with hematoxylin-eosin and reviewed by a pathologist. All tumor slides revealed malignant cells and all slides of surgical margins showed no signs of carcinoma. A second slide was used for immunohistochemical staining.

IMMUNOHISTOCHEMICAL STAINING

Five-micrometer-thick sections from each paraffin-embedded block were cut and placed on superfrost plus slides. The slides were baked at 60°C for 1 hour, deparaffinized in xylene, and rehydrated in graded solutions of alcohol. Slides were immersed in 4% hydrogen peroxide and 60%-methanol for 30 minutes to remove endogenous peroxide activity, and then permeabilized in 0.1% Triton-X detergent in phosphate-buffered saline. Immunostaining was performed with 1:500 dilution of rabbit anti-eIF4E antiserum (Supersensitive Detection Kit; Biogenex, San Ramon, Calif) was used according to the manufacturer’s instructions using 3-amino-9-ethylcarbazole as the chromogen. Normal epithelium from a patient with Zenker diverticulum was used as a control. Negative controls with an omission of the antiserum from the primary incubation were included.

All slides were evaluated for expression of eIF4E by light microscopy, in a blinded manner, by 2 independent investigators who were unaware of the clinical details. Immunostaining for eIF4E was evaluated in both the primary tumor slides and the margin slides. Positive staining in the primary tumor slide was defined as presence of perinuclear staining in the malignant cells. Positive staining in the margin slides was defined as the presence of perinuclear staining in the basal layer cells of the mucosal margins.

Gradation of staining depended on intensity of staining and percentage of cells staining. Scores were ranked as no staining, weak staining, intermediate staining, and strong staining. Values of 0, 100, 200, and 300 were assigned to the intensity of staining. Tumor eIF4E scores were determined by multiplying the intensity of staining value by the percentage of cells staining positive. The details of this semi-quantitative method for reporting immunohistochemical assay results have been described previously. The eIF4E scores were similarly determined for each margin and the margin with the highest score was used for the analysis.

STATISTICAL ANALYSIS

The data on patient characteristics and surgical margins were analyzed statistically with SAS Version 6.07 software (SAS Institute, Cary, NC). Time to recurrence was calculated from surgery to the date of the first documented recurrence. Contingency tables and the χ² test were used to evaluate the association of eIF4E in the surgical margins with tumor size, lymph node status, site, and grade. The Kaplan-Meier method and the log-rank test were used to determine the effects of eIF4E expression in the surgical margin on the time until recurrence. Cox regression analysis was used to test for the effect of each of the different variables in the study (controlling for the other covariates) on time until recurrence. The variables entered in the regression model were age, race, tumor site, tumor size, node status, histological grade of tumor, eIF4E levels in the tumor, and eIF4E levels in the surgical margin. Node status and eIF4E levels in the margins were expressed as 2 categories each (0 and 1), with 0 representing N0 status and no overexpression of eIF4E in the margins, and 1 representing N1, N2, N3 nodal status and eIF4E overexpression in the margins.

RESULTS

Thirty of the patients were male and 1 was female. Patients ranged in age from 49 to 82 years (mean, 63 years). Nineteen patients were white and 12 were black. Five of the tumors occurred in the hypopharynx and 26 were in the larynx. Twenty of the patients had advanced stage III or IV disease and all received postoperative radiation therapy.

eIF4E was overexpressed in the tumors of all 31 patients. The perinuclear staining was seen in the tumor cells with overexpression of eIF4E, whereas no staining was observed in adjacent normal tissues or the negative control obtained from a smoker with a Zenker diverticu-
The perinuclear staining of a tumor is well depicted in Figure 1. A, Squamous cell carcinoma (hematoxylin-eosin, ×20); B, squamous cell carcinoma with intense reddish brown perinuclear staining (anti-eIF4E stain, ×20); C, histologically normal surgical margin in a patient without recurrence (hematoxylin-eosin, ×20); D, histologically normal margin, no recurrence (anti-eIF4E stain, ×40)—note no staining in the basal cell layer; E, histologically normal surgical margin in a patient with a recurrence (hematoxylin-eosin, ×40); and F, histologically normal surgical margin in a patient with a recurrence showing reddish-brown perinuclear staining of the basal layer (anti-eIF4E stain, ×40).

A total of 83 margins were examined for the 31 patients (average number of margins per patient was 2.7). All surgical margins were histologically negative for tumor. Thirteen patients had no expression of eIF4E in the basal cell layer of the surgical margins (Figure 1, C and D) while 18 patients had at least 1 surgical margin overexpress eIF4E (Figure 1, E and F). A comparison of both groups is shown in Table 1. In the eIF4E-negative group, the average disease-free interval was 82.02 months and only 1 patient (8%) experienced a local recurrence. The average eIF4E tumor score was 140 and the surgical margin score was 0, as no detectable level of eIF4E was seen in the basal cell layer. In the eIF4E-positive group, the average disease-free interval was 31.95 months and 12 (67%) of the 18 patients had a recurrence. There were 4 local and 8 regional recurrences. The average eIF4E tumor score for this group was 211 and the average surgical margin score was 20.83.

The Cox regression analysis indicated that eIF4E in the surgical margin (P = .01), nodal status (P = .06), site (P = .02), and age (P = .02) had significant effects on time until recurrence. Nodal status (positive or negative), although not significant at the 5% level, was declared significant since the P value was close to .05. Table 2 presents the outcome of this analysis. It is seen from the risk ratio column that the hazard of recurrence for a patient with eIF4E in the margin was 5.94 times that of a pa-
tient with no eIF4E expression provided that the 2 patients were alike with regard to the other variables in the Table. Similarly, the hazard of recurrence for positive node status was 3.34 times that for negative node status. Also, the hazard of recurrence for hypopharynx tumor was 4.9 times that for tumors of the larynx. In the case of age, for each 1-year increase in age, the hazard of recurrence increased by an estimated 9.8%.

The Kaplan-Meier method using the log-rank test showed that the estimated probability of no recurrence at a given time in months for eIF4E-positive and eIF4E-negative margins were significantly different (P = .002). These are presented in Figure 2.

Table 2 presents comparisons of the probability of no recurrence for the 4 categories (eIF4E-positive and eIF4E-negative margins with positive and negative nodal status). The log-rank test indicates that the probability curves were heterogeneous (P < .001). The eIF4E-positive margin and positive node class gave the smallest probability estimates. The contingency chi-square analysis was performed to determine whether eIF4E in the margins was associated with tumor size, nodal status, site, grade, or postoperative radiation. The analysis showed that only eIF4E in the margins and postoperative radiation were associated (P = .047).

Research in head and neck cancer has recently focused on the role of 2 types of genes involved in the regulation of the cell cycle: proto-oncogenes and tumor-suppressor genes. The p53 tumor suppressor gene is one of the most common genetic alterations in HNSCC. Sixty percent of primary SCC of the larynx displayed nuclear p53 overexpression as revealed by immunostaining with monoclonal antibody. Detection of p53 mutations in histologically negative surgical resection margins was found to predict local recurrence. However, not all SCC tumors are p53-positive; hence, the incidence of detecting positive margins with p53 would be even less. The proto-oncogene eIF4E is elevated in all HNSCC analyzed by Western blot and not in benign lesions that show normal levels of expression, making eIF4E a useful tumor marker.

In this study we used immunohistochemistry to assess eIF4E expression in the specimens. Immunohisto-
chemical staining has advantages over Western blots. Histological correlation can be obtained and it has the potential for use during surgery as an adjunct to standard hematoxylin-eosin stains. Consistently elevated expression of eIF4E was detected in all 31 tumors analyzed. The average score of eIF4E in tumors of patients who had a recurrence was 216, while patients without a recurrence had an average eIF4E tumor score of 156. However, the colorimetric staining in the tumor plausus out because of a saturation effect. Therefore, a more accurate and objective method of quantitating levels in the tumor would be Western blot analysis or immunofluorescence, a technique that continues to be optimized. The immunohistochemistry method did allow easy detection and quantitation of the surgical margins due to the normal absence of eIF4E in the basal layer of the margins. Tumors that incited a strong desmoplastic response stained more intensely than the nondesmoplastic tumors. eIF4E plays a role in tumor angiogenesis through formation of stromal elements and this may explain why tumors that incite a desmoplastic response have a higher expression of eIF4E. It may be that eIF4E causes a higher stromal response to recruit elements to cause increased vascularization.

eIF4E has been shown to play a role in tumor progression by inciting tumor angiogenesis through the translational increase of both fibroblast growth factor 2 and vascular permeability factor.8

Immunohistochemical staining of normal mucosa from the head and neck using Zenker diverticulum and mucosa from the soft palate of a patient who underwent an uvulopalatopharyngolasty showed that eIF4E staining was absent in the basal germinative layer of the epithelium. There appeared to be nonspecific diffuse staining of the superficial layers. The basal layers of the histologically negative surgical margins did not show any perinuclear staining for eIF4E. However, the histologically negative surgical margins that did stain positive for eIF4E in the basal layers of the mucosa correlated with local-regional recurrence. It is interesting that eIF4E was not elevated in the basal cell layer, which is the germinative layer of the mucosa, showing that eIF4E is not simply a marker for proliferating cells. Some margins displayed atypia. However, only a few stained positive for eIF4E, and, again, these were in the patients who developed a recurrence. Hence, it was not morphologic changes such as dysplasia, but rather eIF4E positivity, that was an important factor in determining recurrence, suggesting that cancer cells may be missed when they appear normal or that eIF4E overexpression may denote a very early step in malignant transformation.

Head and neck surgeons rely on the standard histopathological assessment of surgical margins to ensure total excision of the tumor. There is a high rate of local recurrence in HNSCC, occurring in up to 50% of patients with microscopically negative surgical margins.23-25 Current techniques may not detect small numbers of cancer cells at the margins of resection or cells that may promote the process of tumorigenesis by inciting stromal elements and vascular endothelia required for tumor progression. The failure rate in the treatment of HNSCC is based largely on local recurrence from inadequate resection. The process of malignant transformation begins at the molecular level prior to any phenotypic abnormalities. The ability to detect these molecular changes at the time of surgery may aid in more complete resection, thus decreasing local recurrence rates.

The Cox regression analysis showed that eIF4E-positive margins (controlling for the other variables) significantly increased the risk of recurrence. Node status alone was marginal in significance. The most striking observation was the high probability of recurrence for an individual patient with the combination of eIF4E-positive margin status and lymph node metastases (Figure 3). Differences among the other 3 curves in Figure 3 may not be as meaningful because of the small number of observations (ranging from 3 to 10) within each of the 4 classes.

To our knowledge, this is the first study in which immunohistochemistry has been used to correlate recurrence with overexpression of eIF4E in tumors and surgical margins of head and neck cancers. Somewhere in the multistep process of tumorigenesis, elevation of eIF4E is a necessary event in the progression of solid tumors. The ability to detect this progression at the molecular level in the surgical margins of patients with HNSCC may allow improved survival in these patients by decreasing the local recurrence rate. This will enable improved treatment outcomes by further resection during surgery or including adjuvant postoperative therapy.

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