Overexpressed eIF4E Is Functionally Active in Surgical Margins of Head and Neck Cancer Patients via Activation of the Akt/Mammalian Target of Rapamycin Pathway


ABSTRACT

Purpose: Overexpression of eIF4E in surgical margins of head and neck cancer patients is an independent risk factor for recurrence. We hypothesize that overexpressed eIF4E is functionally active in tumor margins through activation of the Akt/mammalian target of rapamycin (mTOR) pathway.

Experimental Design: Western blots and/or immunohistochemistry were performed to determine whether phosphorylation of mTOR and activation of its downstream molecules eIF4E-binding protein-1 (4E-BP1) and p70 S6 kinase and the upstream modulator of mTOR, Akt, were expressed in margins overexpressing eIF4E.

Results: There was a significant association between phospho-4E-BP1 and eIF4E expression of a margin or a significant difference in phospho-4E-BP1 expression between eIF4E-positive and -negative margins (P < 0.01). A significant association between eIF4E and phospho-p70 S6 kinase as well as eIF4E and phospho-mTOR was also noted (P < 0.05). Western blot analysis indicated a highly significant difference in the phosphorylation status of 4E-BP1 between tumors and resection margins. A total of 89% of the 4E-BP1-expressing margins expressed more of the phosphorylated (β, γ, and δ) isoforms, whereas 81% of the 4E-BP1-expressing tumors expressed more of the unphosphorylated α isoform. A similar difference in Akt activation was noted between eIF4E-positive margins and tumors (P < 0.05).

Conclusions: Overexpression of eIF4E is functionally active in tumor margins through activation of the Akt/mTOR signaling pathway. The greater degree of expression of downstream targets and upstream regulators of mTOR in margins compared with the tumors indicates preferential activation of the Akt/mTOR signaling pathway in margins overexpressing eIF4E. Rapamycin analogs can potentially be used as adjuvant therapy for patients with eIF4E-positive margins.

INTRODUCTION

eIF4E-binding protein-1 (4E-BP1) belongs to a family of repressor proteins that bind to eIF4E and inhibit cap-dependent translation. The binding to eIF4E is determined by the phosphorylation state of 4E-BP1. Hyperphosphorylated 4E-BP1 does not bind to eIF4E. The activated mammalian target of rapamycin (mTOR) phosphorylates 4E-BP1, releasing bound eIF4E to bind to eIF4G, stimulating cap-dependent translation (1). Hence, pharmacological inhibitors of mTOR can sequester eIF4E, consequently inhibiting translation of factors related to tumor progression. Rapamycin inhibits mTOR when it is bound in a complex with FK506-binding protein FKBP-12. The cellular and biochemical effects of rapamycin are believed to be a result of inhibition of mTOR signaling (2, 3) because the interaction of the FKBP-12 complex with mTOR is highly specific. mTOR activation can be assessed by examining phosphorylation levels of its downstream effectors 4E-BP1 (4, 5) and p70 S6 kinase.

Signaling pathways upstream and downstream of mTOR appear dysregulated in human cancers, suggesting that this pathway plays an essential role in maintaining the transformed phenotype and potentially sensitizes these cancers to rapamycin. Oncogenes, including overexpressed receptor tyrosine kinases and constitutively activated mutant receptors, activate type 1A phosphatidylinositol 3’-kinase (PI3K)-mediated signaling pathways (6–8). Additional alterations of the PI3K-mTOR pathway in human cancers include amplification of the p110 catalytic subunit of PI3K, loss of PTEN phosphatase function, amplification of Akt2, mutations of tuberous sclerosis genes (TSC1/2), and overexpression or amplification of eIF4E or S6K1. Furthermore, inactivating mutations in TSC2 regulatory genes occur in cancer-prone Peutz-Jeghers syndrome patients.

Histological diagnosis may be inadequate in determining “tumor-free” margins because malignant transformation at the molecular level may precede phenotypic changes. Most tumor markers identified to date lack the sensitivity required for routine clinical use. We have shown that the proto-oncogene eIF4E is elevated in 100% of head and neck squamous cell carcinomas (HNSCCs; Ref. 9). Overexpression of eIF4E in histological “tumor-free” surgical margins results in a significant increased risk of recurrence compared with patients with eIF4E-negative margins and is an independent predictor of recurrence (10, 11). We hypothesize that overexpressed eIF4E is functionally active...
in tumor margins through activation of the Akt/mTOR pathway. Thus, we wanted to determine whether markers upstream and downstream of eIF4E are expressed in eIF4E-positive margins with the intent of targeting the mTOR pathway as adjuvant therapy for patients with eIF4E-positive margins.

Our preliminary data suggest that the Akt/mTOR pathway is activated in histologically normal tumor margins overexpressing eIF4E. Thus, our focus is on understanding the significance of this not only in predicting tumor recurrence but also in targeting the mTOR pathway for adjuvant therapy.

**MATERIALS AND METHODS**

**Samples and Controls.** Patient tumor and margin samples that had already been analyzed in previous reports for overexpression of eIF4E were used in this study. These patients had all been treated at the Department of Otolaryngology/Head and Neck Surgery (Louisiana State University Health Sciences Center) and the Overton-Brooks Veterans Administration Medical Center (Shreveport, LA). The study was approved by the institutional review board, and informed consent was obtained.

For Western blot and immunoprecipitation analysis, tumor and adjacent nonmalignant mucosa from the surgical margins obtained in the operating room immediately after resection of the primary tumor were frozen in liquid nitrogen and stored at −80°C. For immunohistochemical (IHC) analysis, paraffin-embedded blocks from the tumor and the histologically “tumor-free” surgical margins were obtained, and 5-μm-thick sections were cut and mounted on polylysine-coated slides. Positive controls were tumors from HNSCC patients who overexpressed eIF4E and were also found to overexpress phospho-4E-BP1 and phospho-mTOR. Negative controls with an omission of the antiserum from the primary incubation were included. There were significantly more samples analyzed by immunohistochemistry compared with the number analyzed by Western blots due to the limited protein available for both Western blot and immunoprecipitation. Hence, there was a discrepancy in the number of samples analyzed by different techniques.

**Protein Extraction.** Patient samples (~5 mg) were homogenized for 30 s on ice with Polytron Homogenizers PT 1200C (5-mm diameter; Brinkmann, Westbury, NY). The tissue pellets were suspended in 200 μl of lysis buffer [1× protease inhibitor mixture (Roche Molecular Biochemicals) in 1× cell lysis buffer (Cell Signaling, Beverly, MA), which consisted of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM Na3PO4, 1 mM β-glycerophosphate, 1 mM Na2VO4, and 1 μg/ml leupeptin]. The suspension was placed on ice for 30 min and centrifuged at 14,000 rpm for 30 min at 4°C, and the supernatant was then stored at −80°C. Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL) against a standard graph of known BSA protein dilution.

**Western Blot Analysis for eIF4E, 4E-BP1, and Phospho-p70 S6 Kinase.** For analysis of eIF4E, 4E-BP1, and phospho-p70 S6 kinase, equal amounts of tissue extract (30 μg of protein) were analyzed on a 12% SDS-PAGE gel with Laemmli buffer. The proteins were transferred to an Immobilon-P membrane (Millipore, Bedford, MA) and blocked with 5% nonfat milk in TBST [0.05% Tween 20, 10 mM Tris-HCl (pH 8.0), and 150 mM NaCl] for 1 h. Membranes were incubated overnight with the following antibodies at the dilutions indicated: mouse monoclonal anti-eIF4E antibody (BD Transduction Laboratory, Palo Alto, CA), 1:1000; rabbit polyclonal anti-4E-BP1 antibody (Cell Signaling), 1:500; and rabbit polyclonal anti-phospho-p70 S6 kinase antibody (T421/S424; Cell Signaling), 1:250. The membranes were washed four times with TBST for 5 min each. The secondary goat antirabbit and antimouse IgG alkaline phosphatase conjugates (Bio-Rad, Hercules, CA) were then added at a 1:1000 dilution for 1 h, and membranes were developed using alkaline phosphatase color development reagent (nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl phosphate) for eIF4E and 4E-BP1 (Promega, Madison, WI). Phospho-p70 S6 kinase was developed with a secondary anti-rabbit antibody conjugated to horseradish peroxidase. Proteins were detected using enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

**Akt Immunoprecipitation Assay.** Akt immunoprecipitation assay was performed according to the Akt Kinase Assay Kit protocol (Cell Signaling) by immunoprecipitation of total Akt in tumors and margins of HNSCC patients, and then Akt-induced phosphorylation of glycogen synthase kinase (GSK)-3 (downstream target of Akt) was detected by Western blots using phospho-GSK-3α/β (S21/9) antibody. In brief, 200 μl of cell lysate (500 μg of total protein) were incubated with 20 μl of immobilized Akt antibody slurry for 2–3 h at 4°C and then centrifuged at 14,000 rpm for 4 min. The pellets were washed with 500 μl of lysis buffer, followed by 500 μl of kinase buffer on ice, and then suspended in 40 μl of kinase buffer with 200 μM ATP and 1 μg of GSK-3 fusion protein incubated at 30°C for 30 min. The reaction was terminated with 20 μl of 3× SDS sample buffer mix and centrifuged for 2 min. Triplicate samples consisting of 20 μl of denatured protein were electrophoresed by 12% SDS-PAGE as described above. A rabbit polyclonal IgG antibody for phospho-GSK-3 α/β (S21/9) was then added at a 1:1000 dilution overnight at 4°C (Akt Assay Kit; Cell Signaling) and developed with alkaline phosphatase.

**IHC Staining for eIF4E, Phospho-4E-BP1, and Phospho-mTOR.** IHC staining for eIF4E was performed as described previously (12). Recurrence in HNSCC patients occurred only when eIF4E was overexpressed in the basal cell layer. Phosphorylation of 4E-BP1 by mTOR on Thr37 and Thr46 appears to prime 4E-BP1 for subsequent phosphorylation at Thr70 (4), and hence a rabbit polyclonal antibody to phospho-4E-BP1 (Thr70) antibody was used at a 1:50 dilution for 30 min. We also wanted to determine whether phosphorylation of mTOR in our patient samples was a result of activation of Akt. Hence, a rabbit polyclonal antibody to phospho-mTOR (Ser2448; Cell Signaling) was used at a similar concentration (1:50) for 30 min.

**Statistical Analysis.** To determine association between phospho-4E-BP1, phospho-mTOR, and eIF4E status of a margin, we used a general estimating equation method to account for correlations among margins within patients because each of the 27 patients had more than one tumor margin, depending on the extent of surgical resection. The eIF4E status of a margin was used as possible predictor for phospho-4E-BP1 expression of a margin in the general estimating equation model. Fisher’s exact test was used to compare the phosphorylation status of
4E-BP1, expression of p70 S6 kinase, and the activity of Akt between tumors and margins that were positive for both elf4E and 4E-BP1 in the Western blot and immunoprecipitation analysis, respectively.

RESULTS

Samples from 27 patients were examined. If a single margin was positive for elf4E, then that patient was considered to have an elf4E-positive margin. Twenty-one patients were male, and six were female. Patient age ranged from 44 to 71 years (mean age, 58 years). There were 14 white and 13 African-American patients. The tumor sites varied, with 2 tumors in the hypopharynx, 18 in the larynx, 5 in the oral cavity, and 2 in oropharynx. Twenty-three patients had advanced stage III or IV disease, and all patients received postoperative radiation therapy. Four patients were diagnosed with early-stage disease. Nineteen patients had at least one elf4E-positive margin, and in eight patients, all margins were elf4E-negative. Although tumor material from 27 patients was analyzed, not all margins from all patients could be analyzed for the various markers (except elf4E) because of the limited amount of tissue obtained from the tumor margins. Hence, it was not possible to analyze the whole range of molecular markers in the Akt/mTOR pathway on all samples. For IHC assay with phospho-4E-BP1 and phospho-mTOR, 27 tumors with 111 corresponding margins were available (average, 4 margins/patient). Although 26 tumors and corresponding margins were analyzed for Western blot analysis with 4E-BP1 antibody, for p70 S6 kinase we had sufficient protein for only 16 tumors and margins. Immunoprecipitation for Akt required even larger protein samples, and hence only 16 tumors and margins were analyzed. Patient margin samples were very small, making it difficult to obtain enough protein for these various markers, unlike immunohistochemistry, in which the same paraffin-embedded block that was used by the pathologist to analyze H&E-stained margins was used to cut more sections for IHC staining.

4E-BP1 Is Hyperphosphorylated in the Surgical Margins of Tumor Specimens. Western blot analysis was performed on 26 available HNSCC tumor samples that were positive for elf4E expression. One of the tumors from the 27 patients was too small to obtain enough protein because this patient had a T1 lesion of the larynx. All 26 tumors expressed 4E-BP1. In addition, 16 elf4E-positive (elf4E-overexpressing) margins and 10 elf4E-negative (normal elf4E) margins from the corresponding tumor samples were selected from our previous pool of margins and analyzed for 4E-BP1 expression. Nine of the 16 elf4E-positive margins were also positive for 4E-BP1, whereas all 10 elf4E-negative margin samples did not express 4E-BP1. We then analyzed the phosphorylation status of 4E-BP1-expressing tumors and margins by comparing the α, β, γ, and δ isoforms [the Greek letters are arbitrary designations for immunoreactive bands that represent different phosphorylated forms of 4E-BP1 (Fig. 1)]. The fastest-migrating isoform (the α isoform) is unphosphorylated, whereas the other, slower-migrating isoforms have varying degrees of phosphorylated isoforms of 4E-BP1 (4). Fig. 1 is a Western blot showing pairs of alternating bands of tumors (T) and corresponding margins (M). elf4E was present in all four tumors (samples 27, 28, 2, and 22) but in the margins of only two samples (samples 2 and 22). Similarly, 4E-BP1 was phosphorylated in all four tumors, but it was phosphorylated only in the two margins positive for elf4E. There were obvious qualitative and quantitative differences in phosphorylation between tumors and margins. Additionally, there were differences in the 4E-BP1 isoforms between the tumors and positive margins. The tumors showed more of the unphosphorylated α isoform (bottom band), and the corresponding positive margins displayed the phosphorylated β, γ, and δ isoforms but lacked the unphosphorylated α isoform. Interestingly, when all patient samples were analyzed, 89% (8 of 9 samples) of 4E-BP1-expressing margins expressed more of the phosphorylated γ isoform, whereas 81% (21 of 26 samples) of the 4E-BP1-expressing tumors expressed the unphosphorylated α isoform. Fisher’s exact test shows a highly significant differ-
ence in the degree of phosphorylation between the 4E-BP1-expressing margins and tumors [8 of 9 margins (89%) versus 5 of 26 tumors (19%); *P < 0.01]. Hence, it appears that the downstream target 4E-BP1 of mTOR is activated in patients with eIF4E-positive margins, and it is the phosphorylated γ isoform of 4E-BP1 that predominates. However, the level of mTOR activation, as determined by the phosphorylation of 4E-BP1, appears to be lower in tumors compared with the tumor resection margins.

p70 S6 Kinase the Other Downstream Target of mTOR Activation Is Expressed in eIF4E-Overexpressing Margins. Availability of protein limited analysis of expression of phospho-p70 S6 kinase to 16 tumors and the corresponding margins. Twelve of the 16 tumors (75%), all of which overexpressed eIF4E, also expressed phospho-p70 S6 kinase. This pattern is seen in Fig. 1, in which 27-T, 28-T, and 22-T express phospho-p70 S6 kinase. However, a pattern similar to the differential expression of 4E-BP1 was noted between tumors and eIF4E-positive resection margins. Although 75% of the tumors expressed phospho-p70 S6 kinase, the margins overexpressing eIF4E had higher expression of phospho-p70 S6 kinase compared with the tumors themselves (Fig. 1, samples 2 and 22). Conversely, when tumors were compared with eIF4E-negative margins, possibly indicating tumor-free mucosa at the molecular level, tumors had higher expression of phospho-p70 S6 kinase (Fig. 1, samples 27 and 28). In the entire group of 16 patients, there was a significant association between eIF4E and phospho-p70 S6 kinase expression in the margins. eIF4E-positive margins had significantly higher phospho-p70 S6 kinase expression rates of [7 of 8 (87.5%)] compared with the eIF4E-negative margins [2 of 8 (25%); *P = 0.041, Fisher’s exact test].

Phosphorylation of 4E-BP1 and p70 S6 Kinase Is Correlated with Akt Activity. The well-documented evidence indicating that mTOR is downstream of both PI3K and Akt (13) led us to determine whether phosphorylation of mTOR on Ser2481 was a result of Akt activation (13–15). Activation of Akt was demonstrated by assessing the phosphorylation of GSK-3. A pattern of phosphorylation of GSK-3 was seen in tumors and margins similar to that observed with 4E-BP1. Sufficient protein was available to allow analysis of seven eIF4E-positive tumors and the corresponding margins (five of which were eIF4E-positive) for activity of Akt. Using Fisher’s exact test to compare Akt kinase activity between tumors and margins that were positive for eIF4E, we observed a significant difference in Akt kinase activity among the eIF4E-positive margins compared with the eIF4E-positive tumors [4 of 5 (80%) versus 1 of 7 (14%); *P < 0.05]. Fig. 2 presents two representative patient samples of tumors (42T and 50T) and the corresponding margins (42M and 50M). Both tumor samples demonstrate the presence of eIF4E and predominance of the unphosphorylated α isoform of 4E-BP1. Akt kinase activity in tumors indicated by the band labeled Phospho-GSK-3α/β (Ser 21/9) corresponded with eIF4E expression in the tumors. Patient margin 42M, an eIF4E-positive margin, shows expression of the γ phosphorylated isoform of 4E-BP1 and higher expression of Akt kinase activity than the tumor, as noted by the increased expression of phospho-GSK-3α/β. Patient margin 50M, an eIF4E-negative margin, does not express phospho-4E-BP1 and shows lower expression of phospho-GSK-3α/β than the corresponding tumor (50T).

Phosphorylation of 4E-BP1 by Immunohistochemistry Colocalizes with eIF4E Expression in Surgical Margins. We evaluated the expression of phospho-4E-BP1 (Thr70) by immunohistochemistry in 111 margins from the 27 HNSCC patients described above. All 27 tumors expressed both eIF4E and phospho-4E-BP1. Of the 111 margins, 40 were positive for eIF4E, which is determined by expression of eIF4E in the basal cell layer. Previous studies have shown that because eIF4E is a proto-oncogene, it is present in all cells, and hence staining is present in the superficial layers of the epithelium and the submucosal layers. However, in normal epithelium from noncancer patients and in margins from patients who have not suffered disease recurrence, the basal cell layer does not express eIF4E. Such samples are considered to be eIF4E negative (12, 16). Of the 40 eIF4E-positive margins, i.e., those in which the basal cell layer contained eIF4E, 36 (90%) also expressed phospho-4E-BP1. Seventy-one of the 111 margins were eIF4E negative, and 38 of the 71 margins (54%) expressed phospho-4E-BP1. The highly significant difference in the phospho-4E-BP1 expression rate between the eIF4E-positive and -negative margins (90% versus 54%; *P < 0.01) as indicated by the general estimating equation analysis leads to a conclusion of a significant association between the phospho-4E-BP1 and eIF4E status of margin. This is consistent with our findings using Western blot analysis.

Fig. 3A is an IHC picture of a tumor from a patient with hypopharyngeal squamous cell cancer that was found to overexpress eIF4E and phospho-4E-BP1 (Fig. 3, B and C), as indicated by the reddish-brown perinuclear stain in tumor cells. Phospho-4E-BP1 staining is less intense in the tumor as compared with the positive margin (Fig. 3K). This corroborates our Western blot results with 4E-BP1, in which a greater degree of expression of phospho-4E-BP1 was noted in the tumor margins compared with the tumor core. Fig. 3E is a histologically

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Fig. 2 Akt activities were measured by immunoprecipitation of total Akt in tumors and margins of head and neck squamous cell carcinoma patients, and then Akt-induced phosphorylation of GSK-3 (a downstream target of Akt) was examined by Western blots using phospho-GSK-3α/β (Ser 21/9) antibody. A pattern similar to the differential expression of phospho-eIF4E-binding protein-1 (4E-BP1) was noted between tumors and eIF4E-positive margins. Akt kinase activity is lower in tumor (42T) as compared with the eIF4E-positive margin (42M), as seen in the top panel. This corresponds to the pattern seen with 4E-BP1 phosphorylation in the bottom panel, in which the tumors express more of the unphosphorylated α isoform of 4E-BP1, whereas the eIF4E-positive margins (42M) express more of the phosphorylated γ isoform of 4E-BP1. However, Akt kinase activity is higher in the tumor (50T) when compared with eIF4E-negative margin (50M).
Fig. 3 Immunohistochemical staining of eIF4E, phospho-eIF4E-binding protein-1 (4E-BP1; Thr70), and phospho-mammalian target of rapamycin (mTOR; Ser\textsuperscript{2448}) in squamous cell cancers and “tumor-free” surgical margins of the hypopharynx. A–D, tumor stained with H&E, anti-eIF4E, anti-phospho-4E-BP1, and anti-phospho-mTOR, respectively. Note the intense reddish-brown perinuclear staining in tumor cells, indicating overexpression of eIF4E, phospho-4E-BP1, and phospho-mTOR. E–H, histologically “tumor-free” surgical margin without dysplasia stained with H&E, anti-eIF4E, anti-phospho-4E-BP1, and anti-phospho-mTOR, respectively. Although staining is noted in the superficial and submucosal layers, no perinuclear (cytoplasmic) staining is seen in the basal cell layer (arrow; insets show a higher magnification of the basal cell layer), indicating eIF4E-negative margin without expression of phospho-4E-BP1 and phospho-mTOR. I–L, histologically “tumor-free” surgical margin without dysplasia stained with H&E, anti-eIF4E, anti-phospho-4E-BP1, and anti-phospho-mTOR. Note the reddish-brown perinuclear staining of the basal cell layer (arrow; insets show a higher magnification of the basal cell layer), indicating eIF4E-positive margin with colocalization of phospho-4E-BP1 and phospho-mTOR expression.

Phosphorylation of mTOR by Immunohistochemistry Is Associated with eIF4E Expression in Surgical Margins. Phosphorylation of mTOR at Ser\textsuperscript{2448} is believed to occur through Akt activation (13–15). Because Akt kinase activity was increased in eIF4E-positive margins, we also wanted to determine whether mTOR was phosphorylated in eIF4E-positive margins. All 27 tumors expressed both eIF4E and phospho-mTOR. Of the 99 margins that were available for analysis (3 margins were unavailable in the T1 laryngeal patient who underwent a hemilaryngectomy because margins were limited), 36 were positive for eIF4E, and 33 of 36 margins (92%) also expressed phospho-mTOR. Sixty-three of the 99 margins were eIF4E-negative, and only 41 of 63 margins (65%) expressed phospho-mTOR. The highly significant difference in phospho-mTOR rates between the eIF4E-positive and -negative margins (92% versus 65%; \(P < 0.01\)) indicates a significant association between the eIF4E and phospho-mTOR status of margin.

As seen in Fig. 3D, the tumor also expresses phospho-mTOR. The eIF4E-negative margin in Fig. 3F does not express phospho-mTOR (Fig. 3H shows no reddish-brown perinuclear stain in the basal cell layer but rather a blue color from the hematoxylin stain). Fig. 3L shows colocalization of phospho-mTOR (note the arrow in the basal cell layer) with eIF4E (Fig. 3J) and phospho-4E-BP1 (Fig. 3K) in a histologically “tumor-free” margin.

DISCUSSION

The mTOR inhibitors are a new class of antitumor agents that are currently in clinical trials. Several cell cycle targets downstream of mTOR are dysregulated in human cancers in-
including eIF4E (17), D-type cyclins (18), p27^{kip} (19), and c-myc (20). Studies have shown that eIF4E overexpression in histologically “tumor-free” margins is a significant independent predictor of recurrence (10, 11). Hence, we hypothesize that overexpressed eIF4E is functionally active in tumor margins through activation of the Akt/mTOR pathway. This would have significant implications in targeting the mTOR pathway as adjuvant therapy for patients with eIF4E-positive margins.

Our data show that a significant association exists in eIF4E-overexpressing margins between expression of the downstream targets of mTOR activation (4E-BP1 and p70 S6 kinase) and activation of Akt, as evidenced by mTOR phosphorylation at Ser^{2448}. This association suggests that activation of eIF4E in the margins occurs through activation of the Akt/mTOR pathway. Interestingly, we found a greater degree of activation of the Akt/mTOR pathway in the margins than in the tumor itself. This finding was first observed on the Western blot results, which showed that although all isoforms were expressed in both tumors and margins that overexpressed eIF4E, the degree of phosphorylation of 4E-BP1 was significantly less in tumors compared with the corresponding tumor margins. A similar finding was also noted with phospho-p70 S6 kinase, which was more highly expressed in the eIF4E-positive margins compared with the corresponding tumors. Fortunately, the design of our study allowed for a direct comparison between the tumor resection margin and the tumor itself. Hence, we were able to note the difference in the degree of phosphorylation between the tumor and margins. This difference in activity was also noted when we determined Akt activity upstream of mTOR. We found that eIF4E-positive margins also had higher activity of Akt compared with the tumors. This same phenomenon seen with Akt does show consistency in the difference in kinase activity noted between tumors and margins.

There is controversy as to which site of mTOR phosphorylation reflects mTOR activation. Although Akt phosphorylates mTOR at Ser^{2448} (13–15), the significance of this phosphorylation is not clear because a substitution of Ser^{2448} with alanine does not affect mTOR signaling. On the other hand, Ser^{2481} of mTOR is the autophosphorylation site, and hypoxia inhibited mTOR signaling and prevented Ser^{2481} phosphorylation (21). However, treatment of cells with rapamycin does not affect the autophosphorylation level of Ser^{2481} (22). In our study, we noted increased expression of phospho-mTOR at Ser^{2448} in eIF4E overexpressing margins. Although we attempted to perform immunohistochemistry to assess phospho-mTOR (Ser^{2448}), the available antibodies did not give optimal staining in our tumors (data not shown). mTOR activation is believed to occur as a result of activation of the PI3K/Akt pathway through either overexpression of Akt or loss of the tumor suppressor phosphatase gene PTEN (23). Although a high frequency of mutations of the PI3K/Akt and PTEN pathways (24) has been reported in other malignancies, there have been no reports of mutations of mTOR itself (25, 26). The increase in the activity of mTOR seen in our patients with eIF4E-positive margins, indicated by increased expression of both the downstream targets and upstream modulators of mTOR, suggests the importance of this pathway in tumor cell survival and cell sensitivity to rapamycin compounds (23, 27, 28). Hence, it is attractive to hypothesize that inhibitors of mTOR can reverse these effects and inhibit tumor growth after resection, when tumor cell burden is low.

As noted, activated Akt is thought to phosphorylate mTOR at Ser^{2448} (14). Hence, we also wanted to determine in our eIF4E-overexpressing samples whether phosphorylation of mTOR (Ser^{2448}) was associated with increased Akt activity. In our study with limited patient samples, we did note an increase in Akt kinase activity using GSK-3 as a substrate. The mechanism of activation of Akt is still under investigation in various tumor types. This pathway is constitutively activated in cells with PTEN abnormalities (24). Akt is considered to be a major downstream target for PTEN because many of the suppression effects of PTEN can be avoided by activating Akt. It is intriguing that in our own samples, Akt was not activated in tumors but was activated in the positive margin samples. It is possible that Akt activation in margins could be a result of local concentrations of growth factors and nutrients and not a loss of PTEN function. Another possibility is that Akt is activated in HNSCC through constitutive activation of upstream receptor tyrosine kinases such as epidermal growth factor receptor, which is commonly overexpressed in HNSCC (29, 30).

eIF4E has been found to be overexpressed in a number of solid tumors including breast cancer (31, 32). In breast cancer, a >7-fold overexpression of eIF4E in tumors compared with normal breast tissue was associated with a worse prognosis (33). Although overexpression of eIF4E in margins of HNSCC patients is an independent predictor of recurrence (10); it is not known whether it is functionally active. The activation of eIF4E is dependent on inactivation (i.e., phosphorylation) of 4E-BP1, which belongs to a family of repressor proteins that regulates the activity of eIF4E for translation. The mTOR modulates two separate downstream pathways that control translation of specific subsets of mRNAs including 4E-BP1 and the 40S ribosomal protein S6 kinase (p70 S6 kinase; Refs. 4, 5, 34). Activation of mTOR leads to phosphorylation of 4E-BP1, which then dissociates from eIF4E, allowing eIF4E to bind eIF4G, causing translation initiation of cap-dependent mRNAs (35). Phosphorylation of 4E-BP1 is inhibited by rapamycin, through its action on mTOR, thus decreasing the available eIF4E. Hence, we hypothesize that rapamycin can potentially be used as adjuvant therapy in patients with activation of eIF4E in the margins to possibly sequester the excess eIF4E inhibiting translation of critical cap-dependent mRNAs coding for cell cycle progression (36). There is ample evidence indicating that activation of either PI3K or Akt, both upstream of mTOR, is sufficient to induce the phosphorylation of both 4E-BP1 and p70 S6 kinase through mTOR (37, 38), which we noted in our samples. Furthermore, treatment of activated PI3K- or Akt-expressing cells with rapamycin blocks the phosphorylation of p70 S6 kinase and 4E-BP1, suggesting that mTOR is required for these activities (4, 39). The rapamycin analogs CCI-779 and RAD-001, which have strong antitumor potential and favorable pharmaceutical and toxicological characteristics in preclinical studies, are potential adjuvant therapeutic agents in our study population (25, 40).

In a murine lymphoma model, Akt and eIF4E were shown to promote tumorigenesis and drug resistance by disrupting apoptosis (41). In fact, eIF4E was found to be a potent oncogene in vivo and produced phenotypes consistent with an antiapoptotic gene. Rapamycin was shown to reverse chemoresistance.
Akt/mTOR Pathway Activation in HNSCC Surgical Specimens

Akt/mTOR Pathway Activation in HNSCC Surgical Specimens

in lymphomas expressing Akt, but not in those with other apoptotic defects. Interestingly, eIF4E was found to mimic the action of Akt in tumorigenesis and drug resistance but was unable to confer sensitivity to rapamycin and chemotherapy. Although both tumors and margins in HNSCC patients over-express eIF4E, we have seen that eIF4E is preferentially activated in tumor margins compared with the tumors themselves. Hence, we believe that surgically resected HNSCC patients with molecular positive eIF4E margins, in which eIF4E is functionally active, will be sensitive to rapamycin, unlike the eIF4E-overexpressing tumors with decreased activation of the Akt/mTOR pathway, which, like the murine lymphoma model, may not be sensitive to rapamycin alone. An intriguing question is whether the eIF4E-positive margins represent populations of malignant cells that do not exhibit histological findings of malignancy or are premalignant cells. This issue may be both semantic and practical, when the issue of targeted therapy is raised. The progression from various stages of premalignancy to cancer involves changes in the superficial layers of the epithelium (various grades of dysplasia) to involvement of the basal cell layer (carcinoma in situ) to penetration through the basal cell layer (cancer). In our prospective study determining the prognostic value of eIF4E overexpression in surgical margins, there was an increase in expression of eIF4E (determined by overexpression of eIF4E in the basal cell layer) with various grades of dysplasia. However, eIF4E was elevated even in some normal epithelial margins without any histological dysplastic changes, yet recurrence correlated with overexpression of eIF4E and not the grade of dysplasia (10). We postulate that activation of eIF4E in the basal cell layer determined by activation of the Akt/mTOR pathway, irrespective of the phenotypic changes in the epithelium, determines cells that are destined toward malignant progression. The mechanism underlying the overexpression of eIF4E has not been fully elucidated. Although the gene encoding eIF4E has been cloned, the functional elements are not fully defined (42). In addition, eIF4E cDNA has not been sequenced in tumor biopsies. In this study, we see that the tumor itself has a greater degree of the unphosphorylated isoforms of 4E-BP1 and decreased Akt activation compared with the “tumor-free” margins. Hence, these findings lead us to hypothesize that the margins probably contain premalignant cells that are destined toward tumor progression.

Molecular therapeutics is based on identifying an aberration in tumors to select patients who would benefit from molecular targeting therapy. It has been seen that not all tumors with PTEN mutations have aberrant PTEN function (43); hence, molecular diagnostics are needed to identify patients who would be responsive to the rapamycin analogs. It appears that the downstream effects of this pathway (i.e., eIF4E overexpression) may be a molecular diagnostic marker that could identify patients in HNSCC who would benefit from adjuvant therapy with mTOR inhibitors. Rapamycin analogs cause significant tumor growth inhibition rather than tumor regression (18, 26, 44) in vivo experiments with tumor xenografts. The effects of one such analog, CCI-779, were 1.3 times greater when used in combination with cisplatin, suggesting it should be used as a cytostatic agent rather than a cytotoxic agent (45). However, our studies demonstrating that overexpressed eIF4E in margins is functionally active through activation of the Akt/mTOR pathway lend credence to the design of a novel trial. We hypothesize that the rapamycin analogs may be useful as a single agent in a model of minimal residual disease identified by a molecular target eIF4E in the mTOR pathway, thus incorporating biological studies to select patients for enrollment and define a biologically active dose and surrogate end points of antitumor activity for this cytostatic drug.

We have shown that eIF4E is also a potential therapeutic target because decreasing eIF4E with antisense RNA can decrease the tumorigenic and angiogenic potential of eIF4E-overexpressing cells (46, 47). Overexpression of eIF4E results in the increased translation of mRNAs with long 5’-untranslated regions, many of which encode growth-promoting proteins such as c-myc, cyclin D1, ornithine decarboxylase, basic fibroblast growth factor, and vascular endothelial growth factor (17, 48). In HNSCC and breast and bladder cancer, overexpression of eIF4E was found to facilitate the synthesis of basic fibroblast growth factor (46) and vascular endothelial growth factor (49, 50), both of which are potent angiogenic factors for tumor progression. The difficulties faced with delivery of antisense therapy (51) led us to look at an alternative approach using pharmacological inhibitors as adjuvant therapy for patients with eIF4E-positive margins. The findings of preferential activation of the Akt/mTOR pathway in our tumor-free margins compared with the tumor itself could be useful in the design of clinical trials with mTOR inhibitors.

REFERENCES

Overexpressed eIF4E Is Functionally Active in Surgical Margins of Head and Neck Cancer Patients via Activation of the Akt/Mammalian Target of Rapamycin Pathway


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