Local Recurrence in Head and Neck Cancer: Relationship to Radiation Resistance and Signal Transduction


ABSTRACT

Purpose: Locoregional recurrence is the dominant form of treatment failure in head and neck (H&N) cancer. The epidermal growth factor receptor (EGFR) is frequently amplified in this disease (<80%) and can lead to activation of phosphatidylinositol-3-kinase (PI3K), both directly and indirectly through Ras. We have shown previously that radioresistance could be conferred via the Ras-PI3K pathway. Here we investigate the contribution of EGFR to this pathway and its impact on treatment outcome.

Experimental Design: In a series of 38 H&N cancer patients, overexpression of EGFR by immunohistochemical staining was assessed. PI3K signaling was evaluated by staining for phosphorylated Akt (P-Akt), a downstream target of PI3K. Both EGFR and P-Akt were then related to outcome. Radiation survival was determined in the SQ20B cell line, a radioresistant squamous cell line derived from a recurrent laryngeal cancer, after pharmacological blockade of EGFR with Iressa, of Ras by the FTI L744,832, or of PI3K by LY294002.

Results: A significant association was found between P-Akt staining and local control in the patient series. Two-year local control was 100% for patients staining 0–1+ for P-Akt as compared with 70.6% for patients staining 2–3+ (P = 0.04). In our series of 38 H&N cancers, 30 (78.9%) of the specimens were strongly (3+) positive for EGFR, whereas 25 (65.8%) were moderately to strongly (2–3+) positive for P-Akt. Pharmacologically inhibiting EGFR, Ras, and PI3K led to radiosensitization of SQ20B cells.

Conclusions: Evaluation of PI3K activation by Akt phosphorylation might be a prognostic marker for response to therapy, and PI3K could be a useful target for therapy. These results also suggest that signaling from EGFR to PI3K can lead to radioresistance.

INTRODUCTION

Locoregional disease recurrence remains the dominant form of treatment failure for patients with advanced SCC of the H&N. For patients treated with primary radiation therapy, attempts to enhance locoregional disease control have included altered fractionation schemes (1) and incorporation of chemotherapy (2, 3). Although in a select group of patients these strategies result in improvement in local control, treatment toxicity is high.

Sensitivity of tumor cells to radiation therapy is a critical determinant of the probability of local control and, ultimately, of cure (4, 5). Thus, one approach to improving the outcome of therapy depends on determining which factors lead to tumor cell resistance to therapy. Overexpression of the EGFR receptor has been shown to accompany development and growth of malignant tumors, including those of the H&N (6). There is also increasing evidence that high expression of EGFR is associated with aggressive tumor growth and poor clinical outcome in these cancers (7). A number of studies has shown a positive relationship between EGFR expression and tumor resistance to radiation (8). Experimentally, Milas et al. (9) demonstrated in mice with H&N carcinoma xenografts enhanced tumor radiosensitivity after combined treatment with C225 (monoclonal anti-EGFR antibody) and radiation. Similarly, Bonner et al. (10) have shown that combining C225 and radiation results in greater cell killing of SCCs than with either treatment alone. Signaling through the erb family of receptors similarly led to radioresistance in a glioma cell line (11). The results of these studies have provided the basis for proceeding with clinical trials (reviewed in Ref. 12).

The EGFR family consists of four closely related growth factor receptors, including EGFR or HER-1 (erb-B1), HER-2 (erb-B2/neu or p185(neu)), HER-3 (erb-B3), and HER-4 (erb-B4). EGFR binds several distinct ligands, including EGF, transforming growth factor-α, and amphiregulins. Heregulins and neu...
regulins bind to erb-B3 and erb-B4. Erb-B2 (HER-2/neu) does not directly bind to any known ligand. Instead, it forms heterodimers with the three other family members and, in so doing, enhances ligand-binding affinity and reduces the rate of ligand dissociation. HER-2/neu heterodimers also amplify growth factor signals through activation of the HER-2/neu intracellular kinase domain and auto-cross phosphorylation (reviewed in Ref. 13).

The detailed mechanism by which EGFR signaling leads to radiation resistance is unknown. EGFR receptors initiate cytoplasmic signaling through autophosphorylation of their intracellular domains (14). EGFR has a number of effectors that include Ras and PI3K. Transfection with oncogenic ras itself has also been shown to increase radioresistance (15). We have shown that Ras-mediated radiation resistance is mediated through PI3K (16) and that P-Akt was a good marker for this effect. PI3K activity is stimulated by Ras activation as a result of direct interaction (17). PI3K phosphorylates PtdIns-4,5-P2 to yield PtdIns-3,4,5-P3. PtdIns-3,4,5-P3 in turn causes membrane localization of protein kinase B (Akt) and the phosphoinositide-dependent kinase phosphoinositide-dependent kinase 1 (18). Phosphoinositide-dependent kinase 1 phosphorylates one of two sites on Akt (19), whereas a second PI3K-activated kinase, ILK-1, phosphorylates a second site (20), resulting in full activation of Akt. Akt has been shown to act as an inhibitor of apoptosis (21). One mechanism for the antiapoptotic activity of Akt appears to be the phosphorylation and inactivation of the proapoptotic BAD protein, although multiple other proteins are also substrates for Akt phosphorylation (reviewed in Ref. 22). These findings together implicate Akt as a possible regulator of cell survival. Because Akt is downstream of PI3K, Ras, PTEN, and EGFR, they also raise the possibility, explored here, that there might be common pathways mediating radiation resistance in tumors carrying multiple types of oncogenic mutation.

In this study, we asked whether EGFR expression and Akt phosphorylation measured both in human H&N cancers and tissue culture are associated with the response to radiation. If the EGFR-Ras-PI3K pathway mediates radiation resistance in human H&N cancers, then examination of activity in this pathway might predict outcome in these patients. Association between immunohistochemical staining of EGFR, Pan Akt, and P-Akt and clinical outcome was tested in patients with H&N cancer treated similarly with chemotherapy and radiation. We found P-Akt to be a significant predictor for local control, further indicating that the EGFR-Ras-PI3K pathway might play an important role in mediating radiation resistance.

Further exploring this idea using the H&N cancer cell line SQ20B that has constitutively active EGFR and wild-type Ras, we found that Akt was phosphorylated constitutively. Treatment of SQ20B cells with the EGFR inhibitor Iressa, the Ras processing inhibitor, FTI L744,832, or the PI3K inhibitor LY294002 resulted in both reduced Akt phosphorylation and caused radiosensitization.

**PATIENTS AND METHODS**

**Patient Selection.** Fifty-three patients with advanced (Stage III/IV) oropharyngeal cancer were treated in a Phase II trial (23) at the University of Pennsylvania between August 1997 and June 2000. All patients were treated by the same team of physicians. The patients were treated with combined modality therapy consisting of induction chemotherapy with carboplatin and paclitaxel followed by chemoradiotherapy with planned neck dissection for N2 disease or greater. If patients did not have at least a partial response to induction chemotherapy, they were referred for radical surgery followed by postoperative radiation. Additional treatment details and results have been published previously (23). Of the 53 patients originally entered in this trial, we were able to obtain sufficient paraffinized tissue from 38 of the 53 patients for immunohistochemical staining. In all cases, the tissue was from the time of original diagnosis and before any treatment. All patients were followed at 1–2 month intervals by the radiation oncologist, surgeon, and the medical oncologist. Follow-up for survival is through June 2001.

**Immunohistochemical Staining.** Paraffin-embedded tissue sections were stained with antibody to total EGFR clone H11 (DAKO Corp.), using the DAKO Envision + System, and IHC-specific phosphorylated Ser 473 Akt and pan Akt antibodies (New England Biolabs), as described by Zhou et al. (24). Immunohistochemically stained slides were interpreted blindly and independently by two pathologists (M. D. F. and J. D. G.) using a four-tiered grading system based on staining pattern and intensity. Grading was based on examination of invasive tumor only. Because keratinized tumor cells often caused artifactual staining, these keratinized areas were excluded from the analysis. EGFR-stained slides were graded as negative, 1+, 2+, and 3+ based on the intensity of the membrane staining and pattern. If the general staining was weak with a pattern of incomplete circumferential staining of each tumor cell, this qualified as 1+. The 2+ showed complete circumferential staining with a weak or intermediate intensity, and 3+ showed complete circumferential staining with strong intensity. Pan Akt and P-Akt immunohistochemical stains were interpreted using identical grading schemes. The 1+ pattern was defined as weak, homogeneous cytoplasmic positivity without a granular staining pattern. The 2+ and 3+ patterns both had strong granular cytoplasmic staining with the 2+ having it in <20% of the tumor cells and the 3+ having it in >20% of the tumor cells.

**Statistical Consideration.** Descriptive statistics were used to characterize the distribution of patient variables. For categorical variables, frequency and percentage were used. For continuous variables, mean, SD, minimum, and maximum were used. The association between EGFR and P-Akt was tested by Fisher’s exact test (25). Survival and time to local failure distributions were estimated by the method of Kaplan and Meier (26). Survival was defined as months from diagnosis to death because of any cause or last patient contact. Time to local failure was defined as months from diagnosis to documented local failure. Patients experiencing other events, such as distant failure or death without documented local failure, were censored. Survival and time to local failure were compared between groups of patients by the Log-rank test (27). The degree of agreement between the two pathologists classifying the same sample using the same ordered scale (e.g., 0–3 scale for EGFR and P-Akt) was assessed by a weighted κ statistic (28). A one-sided Exact Linear trend test for ordered populations (StatXact v. 4.0) was used to look at T Stage and P-Akt positivity and local failure. All Ps quoted are two sided. A P of
<0.05 is considered statistically significant. Statistical analyses performed in either StatXact v. 4.0 (Cytel Corp., Cambridge, MA) or SPSS v. 9.0 (SPSS, Inc., Chicago, IL).

Cells. SQ20B cells were obtained from American Type Culture Collection (Rockville, MD). Cells were cultured in DMEM (Fisher Scientific, Pittsburgh, PA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), penicillin (100 units/ml), and streptomycin (100 mg/ml; Life Technologies, Inc., Gaithersburg, MD) at 37°C in humidified 5% CO₂–95% air.

Inhibitors. The PI3K inhibitor LY294002 and the mitogen-activated protein/extracellular signal-regulated kinase inhibitor PD98059 were obtained from Alexis Corp. The EGFR inhibitor Iressa was obtained from Astra Zeneca. The FTI L744,832 was obtained from Merck Pharmaceuticals. All inhibitors were dissolved as concentrated stock solutions in DMSO and diluted at the time of treatment in medium. Control cells were treated with medium containing the same concentration of DMSO.

Cell Growth Curves. Cultures in log growth phase were counted, and 3 × 10⁵ cells were plated in each T25 flask. The cells were allowed to attach, and the inhibitors were added. At various times, total cell number was assessed in triplicate.

Radiation Survival Determination. Cultures in log growth phase were counted and plated in 60-mm dishes containing 2 ml of media. The cells were allowed to attach, and inhibitors were added to cultures ≥1 h before radiation. L744,832 treatment was initiated 24 h before radiation. Treatment was continued for 24 h after irradiation, at which time 3 ml of additional drug-free media were added to the kill curves involving LY294002, PD98059, and L744,832. For the survival curves with Iressa and the control for the Iressa-treated curves, the media were completely replaced at 24 h with fresh media so no Iressa remained in the dishes. Cells were irradiated with a Mark I cesium irradiator (J. L. Shepherd, San Fernando, CA) at a dose rate of 1.6 Gy/min. Colonies were stained and counted 10–14 days after irradiation. A light box was used to assist in counting colonies. The surviving fraction was calculated by dividing the number of colonies formed by the number of cells plated times plating efficiency. Each point on the survival curve represents the mean surviving fraction from at least three replicates.

Western Blotting. Cells were lysed without trypsinization by rinsing culture dishes once with PBS followed by lysis with reducing Laemeli sample buffer. Samples were boiled, sheared, and clarified by centrifugation and stored at -80°C. Samples containing equal amounts of protein were separated on 12% SDS polyacrylamide gel and blotted onto nitrocellulose membranes. Membranes were blocked in PBS containing 0.1% Tween 20 and 5% powdered milk before primary antibody addition. Monoclonal H-Ras antibody LA069 (Quality Biotech) was used at a dilution of 1:5000; monoclonal antiphosphorylated EGFR (HER-1; Upstate Biotechnology), polyclonal antiphosphorylated Ser 473 Akt, and polyclonal pan Akt (New England Biolabs) were all used at a 1:2000 dilution. Antibody binding was detected using the enhanced chemiluminescence kit (Amersham, Arlington Heights, IL). Images were digitized using an Arcus II scanner, and figures were assembled using Adobe Photoshop 3.0 and Microsoft Power Point.

Table 1 Patient characteristics

<table>
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<td>Total number of patients</td>
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<td>T3</td>
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<td>T4</td>
<td>16</td>
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RESULTS

EGFR and Akt Phosphorylation in Human H&N Cancer. Patient characteristics are described in Table 1. All of the patients had stage III/IV disease with 33 of the 38 having stage IV disease. At the time of analysis, 12 patients had died. The median follow-up for the 26 living patients was 28.2 months. Fig. 1A demonstrates examples of the staining obtained. H&E staining of typical SCCs is shown. The staining distribution is shown in Table 2. The distribution of EGFR staining was 0 (4 patients), 1+ (1 patient), 2+ (3 patients), and 3+ (30 patients). All of the tumors stained using the pan Akt antibody. The distribution for P-Akt staining was 0 (3 patients), 1+ (10 patients), 2+ (16 patients), and 3+ (9 patients). Fig. 2 shows examples of different intensities and densities of staining with P-Akt. In the 30 patients with strongly positive EGFR staining, 20 or 67% had positive P-Akt staining. In the 8 patients with weak or moderate EGFR staining, 5 were positive for P-Akt (Table 3). No association between EGFR and P-Akt was evident (P = 1). The agreement between our two pathologists was very strong; weighted κ statistic was 0.92 for EGFR and 0.71 for P-Akt (P < 0.0001 for each). It should be noted that false positive staining was frequently noted on keratin both in keratin pearls (Fig. 1B) and the epithelium (Fig. 1C) but that this staining was excluded from consideration. In addition, inflammatory cells stained intensely positive for P-Akt and served as an internal control (Fig. 1D). Neither EGFR nor P-Akt staining was associated with T stage, N stage, or differentiation of disease (data not shown). The numbers, however, were too small to do meaningful multivariate analysis. Of the 8 patients that were T2, 5 were P-Akt positive, and 0 had local failure; of the 14 patients that were T3, 8 were P-Akt positive, and 2 or 25% had local failure; of the 16 patients that were T4, 12 were P-Akt positive, and 5 or 42% had local failure (Table 4). Higher T stage was not associated with increased P-Akt positivity (P = 0.29). Higher T stage was associated with increased local failure...
Of the 7 patients with local failure, none had evidence of distant disease at the time of relapse. EGFR and P-Akt Correlated to Outcome. At the time of this analysis, 26 of the 38 patients (68.4%) were alive. The median survival time for all patients was 41.2 months (95% confidence interval, 26.9–55.5 months). Survival was not associated significantly with EGFR (P = 0.41) or P-Akt (P = 0.16) staining (Fig. 3). There was a total of seven local failures in this group. All of the local failures were in the EGFR-positive (3+) and P-Akt-positive (2–3+) groups. Time to local failure was significantly associated with P-Akt (P = 0.04; Fig. 4B). EGFR expression was not a statistically significant predictor of time to local failure (P = 0.14; Fig. 4A), but it should be noted that there were 8 EGFR-negative to moderate staining specimens in this series.

Tissue Culture Analysis. Our data on this patient group raise the possibility that evaluation of signaling through this pathway might predict treatment outcome. This led us to study the H&N cell line SQ20B. SQ20B is a human H&N cancer cell line with a constitutively active mutation in EGFR and wild-type ras. This cell line was derived from a locally recurrent laryngeal cancer and is highly radioresistant. The activity of the EGFR-Ras-P13K-Akt signaling pathway was verified in the SQ20B cells. Phosphorylated EGFR was detected in SQ20B as expected from its active EGFR. The downstream target Akt was also phosphorylated (Fig. 5). Inhibition of EGFR with Iressa (1 μM) resulted in reduced levels of phosphorylated EGFR and P-Akt (Fig. 5). Inhibition of Ras processing by the FTI L744,832 (5 μM) or of PI3K with LY294002 (10 μM) also inhibited Akt phosphorylation.

Clonogenic Survival after Inhibition of EGFR Signaling. To test the effects of EGFR-Ras-P13K signaling on radiation survival, we determined the time course of action for a single application of each of the pharmacological agents. Cells treated with Iressa had decreased P-Akt in ≤10 min of application, maximal inhibition by 1 h, and the effect was maintained for 72 h (Fig. 6A). LY294002 resulted in complete absence of P-Akt by 1 h, but at 24 h, P-Akt could again be detected (Fig.

Table 2

<table>
<thead>
<tr>
<th></th>
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<th>2+</th>
<th>3+</th>
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<tr>
<td>EGFR</td>
<td>4 (10.5%)</td>
<td>1 (2.6%)</td>
<td>3 (7.9%)</td>
<td>30 (78.9%)</td>
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<tr>
<td>P-Akt</td>
<td>3 (7.9%)</td>
<td>10 (26.3%)</td>
<td>16 (42.1%)</td>
<td>9 (23.7%)</td>
</tr>
</tbody>
</table>

(P = 0.05). Of the 7 patients with local failure, none had evidence of distant disease at the time of relapse.

EGFR and P-Akt Correlated to Outcome. At the time of this analysis, 26 of the 38 patients (68.4%) were alive. The median survival time for all patients was 41.2 months (95% confidence interval, 26.9–55.5 months). Survival was not associated significantly with EGFR (P = 0.41) or P-Akt (P = 0.16) staining (Fig. 3). There was a total of seven local failures in this group. All of the local failures were in the EGFR-positive (3+) and P-Akt-positive (2–3+) groups. Time to local failure was significantly associated with P-Akt (P = 0.04; Fig. 4B). EGFR expression was not a statistically significant predictor of time to local failure (P = 0.14; Fig. 4A), but it should be noted that there were 8 EGFR-negative to moderate staining specimens in this series.
We have shown previously that inhibition of H-Ras prenylation by FTI treatment is detected after 2–5 h and reaches 50% by 24 h. Inhibition persists for at least 21 h after inhibitor removal (29). Effects on cell growth by these drugs were also examined (Fig. 6B) because clonogenic survival assays depend on the ability of cells to grow and form colonies. Treatment of SQ20B with Iressa caused a pronounced growth delay for 7 days consistent with the time course showing long-term inhibition by Iressa. Treatment with LY294002 revealed an initial lag in growth followed by recovery to a growth rate equivalent to that of control cells.

We designed clonogenic assays to evaluate the effect of blocking signaling in the EGFR pathway on radiation survival. To test the effect of Iressa while limiting the effect of its growth inhibition, we completely replaced the media in these experiments 24 h of irradiation. Fig. 7A shows the clonogenic survival curve of SQ20B cells with and without Iressa (1 μM).

### Table 3

<table>
<thead>
<tr>
<th>P-Akt negative (0–1+)</th>
<th>P-Akt positive (2–3+)</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>EGFR negative (0–2+)</td>
<td>3 (37.5%)</td>
<td>8 (21.1%)</td>
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<tr>
<td>EGFR positive (3+)</td>
<td>10 (33.3%)</td>
<td>30 (78.9%)</td>
</tr>
<tr>
<td>Total</td>
<td>13 (34.2%)</td>
<td>38 (100%)</td>
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*a P = 1 by Fisher’s exact test.*

### Table 4

<table>
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<th>T Stage</th>
<th>No.</th>
<th>P-Akt positive (2–3+)</th>
<th>Local failure</th>
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<tr>
<td>T2</td>
<td>8</td>
<td>5 (62.5%)</td>
<td>0</td>
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<tr>
<td>T3</td>
<td>14</td>
<td>8 (57.1%)</td>
<td>2 (25%)</td>
</tr>
<tr>
<td>T4</td>
<td>16</td>
<td>12 (75.0%)</td>
<td>5 (42%)</td>
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*One-sided Exact Linear trend test for ordered populations.*
Control curves in which media was replaced at 24 h (Fig. 7A) showed greater apparent radiosensitivity than those that did not undergo this manipulation (Fig. 7B). Nonetheless, Iressa treatment reproducibly lowered survival (Fig. 7A). Disruption of Ras signaling using L744,832 (5 μM) and inhibition of PI3K with LY294002 (10 μM) also sensitized SQ20B cells (Fig. 7B) to radiation. To ascertain that all inhibitors did not result in sensitization of SQ20B cells, we also did clonogenic assays with the mitogen-activated protein/extracellular signal-regulated kinase inhibitor PD98059 (25 μM), which did not result in sensitization (Fig. 7B).

DISCUSSION

The results of this retrospective study evaluating EGFR and Akt in H&N cancer patients treated with multimodality therapy have found a significant association between P-Akt and treatment failure. These results implicate P-Akt in radiation resistance because failures were all local. Moreover, these data are strengthened by in vitro studies showing that inhibition of EGFR, PI3K, and Akt radiosensitized the H&N squamous cancer cell line SQ20B.

We have shown previously that PI3K is an important mediator of Ras-induced radiation resistance (16). In the present study, we show that EGFR, which is upstream of PI3K, may also mediate resistance through this common pathway. It should be noted, however, that there was no direct association between EGFR and P-Akt. Of the eight tumors that were EGFR negative, five had P-Akt-positive disease, implying that mutations downstream of EGFR can also result in activation of Akt. Similarly,
EGFR overexpression did not guarantee that the PI3K pathway would be activated, because 10 of the 30 tumors that were EGFR positive were P-Akt negative. In addition to EGFR and Ras, PTEN can also regulate the PI3K pathway. PTEN is a phosphatase that antagonizes PI3K by converting its active product PI(3,4,5)P3 to PI(4,5)P2 (30). In the tumors that were EGFR positive yet P-Akt negative, PTEN may be modulating PI3K activity. Mutations in PTEN that cause it to be functionally inactive are also frequently found in many human cancers. Tumor cells with these mutations may have augmented PI3K activity and, hence, be susceptible to radiosensitization by PI3K inhibition. Wick et al. (31) have shown that PTEN gene transfer in human malignant gliomas sensitized cells to radiation, although in this case, PTEN transfer was associated with growth suppression, which may complicate the interpretation.

The estimated incidence of new H&N cancers in the United States is 41,000 (32). Early stage disease (stages T1-T2 and N0) can be cured with either surgery or radiation therapy alone or with a combination of both. However, more advanced tumors that were T2–4 and N1–3 have a local failure rate of $\geq 50\%$ (33). All patients in this study were locally advanced. The numbers were too small to do a multivariate analysis in terms of EGFR, P-Akt staining, T stage, or N stage. There was no increase in P-Akt positivity with more advanced T stage, yet more advanced tumors did have increased local failure. All of the local failures were in the P-Akt-positive patients. Of the tumors that were P-Akt positive and had local failure, 0% were T2, 25% were T3, and 42% were T4. This points toward P-Akt being a confounding variable along with T stage as a predictor of local failure. All seven of the local failures were isolated local failure without evidence of distant disease. There was not a statistically significant association between P-Akt and survival ($P = 0.16$), but the patient numbers were too small for any conclusion. Identifying a common signal for EGFR, Ras, or PTEN that results in radiation resistance may uncover targets for developing molecular-based radiosensitization protocols for tumors resistant to radiation and, thus, improve the local control that can be obtained after radiation therapy. Additional work is needed to identify the subset of patients that will benefit most from this treatment.

REFERENCES


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