Decreased Lymphangiogenesis and Lymph Node Metastasis by mTOR Inhibition in Head and Neck Cancer

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Abstract

Despite our improved understanding of cancer, the 5-year survival rate for head and neck squamous cell carcinomas (HNSCC) patients remains relatively unchanged at 50% for the past three decades. HNSCCs often metastasize to locoregional lymph nodes, and lymph node involvement represents one of the most important prognostic factors of poor clinical outcome. Among the multiple dysregulated molecular mechanism in HNSCCs, emerging basic, preclinical, and clinical findings support the importance of the mTOR signaling route in HNSCC progression. Indeed, we observed here that the activation of mTOR is a widespread event in clinical specimens of HNSCCs invading locoregional lymph nodes. We developed an orthotopic model of HNSCC consisting of the implantation of HNSCC cells into the tongues of immunocompromised mice. These orthotopic tumors spontaneously metastasize to the cervical lymph nodes, where the presence of HNSCC cells can be revealed by histologic and immunohistochemical evaluation. Both primary and metastatic experimental HNSCC lesions exhibited elevated mTOR activity. The ability to monitor and quantitate lymph node invasion in this model system enabled us to explore whether the blockade of mTOR could impact HNSCC metastasis. We found that inhibition of mTOR with rapamycin and the rapalog RAD001 diminished lymphangiogenesis in the primary tumors and prevented the dissemination of HNSCC cancer cells to the cervical lymph nodes, thereby prolonging animal survival. These findings may provide a rationale for the future clinical evaluation of mTOR inhibitors, including rapamycin and its analogues, as part of a molecular-targeted metastasis preventive strategy for the treatment of patients with HNSCC.

Introduction

With approximately 500,000 new cases per year worldwide (1) and more than 11,000 expected deaths in 2009 in the United States alone (2), head and neck squamous cell carcinoma (HNSCC) ranks sixth among the most common cancers in the world (3). Despite clear advancements in our understanding of cancer as a disease, the 5-year survival rate for HNSCC remains relatively unchanged at 50% for the past 3 decades (2, 3).

Several important factors contribute to this bleak scenario, including late presentation and consequent delay in the diagnosis of HNSCC lesions, concomitant with the limited availability of effective therapeutic options to reduce the morbidity and mortality of advanced HNSCC cases (4–7). In this regard, the head and neck region includes a large fraction of all the lymph nodes of the human body, and with this rich lymphatic system, HNSCC has a high propensity to metastasize to locoregional lymph nodes (5, 8–12). Even in patients with no clinical evidence of lymph nodal metastasis (N0), the incidence of occult metastasis ranges from 10% to 50% (8–11), and the status of cervical lymph node metastasis is often considered the single most important prognostic factor in HNSCCs, with the presence of lymph node involvement decreasing the overall survival rate by nearly 50% (5, 9, 12).

Of interest, among the multiple molecular mechanism dysregulated in HNSCCs, emerging basic, preclinical, and clinical findings support the importance of Akt/mTOR signaling route in HNSCC progression (reviewed in ref. 7). Indeed, activation of mTOR and Akt, the latter acting upstream from mTOR, has been observed in more than 80% of all HNSCC lesions (6) often correlating with poor prognosis (13, 14). The activation of mTOR can result from the enhanced expression and activity of epidermal growth factor receptors that characterize HNSCC (15), as well as by the overexpression or the presence of...
activating mutations in the catalytic subunit of phosphoinositide-3-kinase α (16) or the decreased expression of the PIP₃ phosphatase PTEN (17). Furthermore, interfering with mTOR activity in its complex 1 (mTORC1) by the use of specific inhibitors, such as rapamycin (sirolimus) and RAD001, known as everolimus), has been shown to provoke the rapid regression of HNSCC tumor xenografts (18), to prevent tumor regrowth in a minimal residual HNSCC xenograft model (19), and to decrease tumor burden and the malignant conversion of potential HNSCC precancerous lesions in multiple genetically defined and chemically induced animal models of HNSCC (20–22). These observations prompted us to examine whether mTOR is activated in human HNSCC lymph nodes metastasis and whether blocking mTOR prevents the metastatic spread of primary HNSCC lesions. We show here that the activation of mTOR is a widespread event in clinical specimens of HNSCCs invading locoregional lymph nodes. Furthermore, the prolonged treatment with rapamycin and RAD001 diminished the dissemination of HNSCC cancer cells to the cervical lymph nodes in a newly developed orthotopic HNSCC model, thereby prolonging animal survival. Thus, the use of mTOR inhibitors may represent a novel molecular-targeted approach for metastasis prevention in patients with HNSCC.

Materials and Methods

Chemicals and reagents and cell culture

All chemicals and reagents were from Sigma-Aldrich, unless indicated. UMSCC2 and UMSCC17B cells were cultured as previously described (23, 24) in Dulbecco’s Modified Eagle’s Media supplemented with 10% FBS, at 37°C in 95% air/5% CO₂, and both cell lines underwent DNA authentication (Genetica Laboratories, Inc.) prior to the described experiments to ensure consistency in cell identity.

Establishment and treatment of orthotopic tumor xenografts in SCID/NOD mice

All animal studies were carried out according to NIH-approved protocols (ASP # 10-569), in compliance with the NIH Guide for the Care and Use of Laboratory Animals. Female severe combined immunodeficient (SCID)/NOD mice (NCl), 4 to 6 weeks of age and weighing 18 to 20 g were used in the study, were housed in appropriate sterile filter-capped cages, and fed and watered ad libitum. All cell and animal handling and tumor transplantation into the tongue are described in detail in Supplementary Material. Briefly, all animals bearing orthotopic HNSCC tumors underwent weekly evaluation of the tongue for disease onset, and the observed lesions were assessed for length and width, and tumor volume was determined as described previously (18). Animals were euthanized at the indicated time points and the cervical lymph nodes assessed for evidence of metastases.

Histopathologic and immunohistologic analysis

For histopathology, after fixing, each tongue was cut into 4 sections of approximately the same thickness, following its major axis (20), and tissue processing, immunohistochemical analysis, image acquisition, and staining quantification were conducted as described in Supplementary Material. Masson trichrome staining was carried out on formalin-fixed, paraffin-embedded tissues as previously described (25).

Statistical analysis

One-way ANOVA followed by the Bonferroni or Newman–Keuls multiple comparison tests was used to analyze the differences of tumor mass volume between experimental groups and differences between immunohistochemical quantification of each group. The Mann–Whitney test was used to evaluate differences in total tumor area. Data analysis were done using GraphPad Prism version 5.00 for Windows (GraphPad Software); values of \( P < 0.05 \) were considered statistically significant for each analysis as described in detail in Supplementary Material.

Results

As we have previously reported, the activation of mTOR is a widespread event in HNSCC, as judged by the immunohistochemical analysis of the presence of the phospho-serine ribosomal protein S6 (pS6) in representative human HNSCC tissue sections (Fig. 1A). These tumors are highly angiogenic, as revealed by the use of the vascular endothelial marker CD31 showing blood vessels within the stroma adjacent to the tumoral mass positive for pS6 (Fig. 1A). Most human HNSCC lesions are also highly lymphangiogenic (26), reflected by the presence of intratumoral lymphatic vessels staining positive for lymphatic vessel endothelia receptor 1 (LYVE-1). Indeed, the microvessel densities of vascular and lymphatic vessels were comparable when analyzing consecutive tissue sections of a representative group of HNSCC lesions (\( n = 5 \); Fig. 1B). Of interest, the presence of active mTOR was also clearly observed in the epithelial cells of all human-invaded HNSCC lymph nodes analyzed (\( n = 8 \)); only isolated lymphocytic subpopulations showed cytoplasmic immunoreactivity in normal, non-invaded areas in human lymph nodes (Fig. 1C). Similarly, we also observed elevated levels of the serine 473 phosphorylated form of Akt (pAktS473), a downstream target of mTOR in its complex mTORC2 (27), in most tumor cells from all invaded lymph nodes evaluated (\( n = 8 \); Fig. 1C). All malignant cells displaying elevated pS6 and pAktS473 in invading tumors were of epithelial origin, as revealed by staining adjacent tissue sections for human cytokeratins (Fig. 1C).

To begin addressing whether molecular-targeted approaches could be used to prevent the spread of HNSCC to locoregional lymph nodes, we took advantage of the availability of highly invasive HNSCC cells to develop an orthotopic model of HNSCC metastasis. Few metastatic models are currently available in which lymph node metastases develop, albeit with limited efficiency (28–30). In particular, the evaluation of a large panel of HNSCC-derived cells led to the identification of 2 highly invasive human HNSCC cell lines, UMSCC2 and UMSCC17B. When orthotopically injected into the tongue of SCID/NOD mice, these HNSCC cells grew as highly aggressive tumors, invading the muscle and all surrounding tissues. For example, intraepithelial
Invasion was readily visible under microscopic evaluation (Fig. 2A and B for UMSCC2 cells and Supplementary Fig. S1A and S1B for UMSCC17B). Remarkably, these HNSCC cells also invaded the nerves and local lymph nodes, with visible tumor masses growing inside the lymphatic vessels (Fig. 2C and D and Supplementary Fig. S1C and S1D). These tumors are highly lymphangiogenic, reflecting a characteristic displayed by most human HNSCC lesions (26). Intratumoral lymphatic vessels staining positive for LYVE-1 were visible within the tumoral mass (Fig. 2E and Supplementary Fig. S1E). The adjacent muscle, which has extensive lymphatic networks, served as a positive control. These tumors are also highly angiogenic, as revealed by CD31 staining.

We next injected India ink orthotopically into lateral tongue to visualize the ink particles into the subcapsular area of the draining cervical lymph nodes (Fig. 3A and B). This enabled us to identify lymphatic drainage to 4 to 5 readily resectable cervical lymph nodes. Indeed, the metastatic spread of HNSCC cells growing orthotopically into the tongue could be visualized in hematoxylin and eosin (H&E)-stained lymph node sections as compared with noninvaded lymph nodes (Fig. 3C–E and Supplementary Fig. S2A–S2D). Nearly, all mice in the initial cohorts had at least one or more invaded lymph nodes when sacrificed 40 days after tumor implantation into the tongue (Fig. 3F and Supplementary Fig. S2E). This provided a simple and quantitative approach to examine the yet-to-be-identified factors contributing to lymph node metastasis and to attempt to halt this life-threatening process. Noninvaded lymph preserved their rich cortical network of normal lymphatic vessels (Fig. 3G and Supplementary Fig. S2F).
 Supplementary Fig. S2F), whereas in metastatic lymph nodes, the tumor mass often displaces the lymphatic ducts (Fig. 3H and Supplementary Fig. S2G).

In normal murine oral mucosa and skin, mTOR is activated in the suprabasal layers lacking proliferative capacity, as judged by the accumulation pS6 (Fig. 4A and Supplementary Fig. S3A). In contrast, the tumor area displayed high levels of pS6 throughout (Fig. 4A and Supplementary Fig. S3A). Similarly, the invaded lymph nodes displayed high levels of pS6; however, the staining was not homogeneous, with necrotic areas and their adjacent cells likely harboring lower mTOR activity (Fig. 4B and Supplementary Fig. S3B). Thus, both experimental and human HNSCC metastatic lesions are characterized by the presence of active mTOR pathway. Rapamycin and RAD001, which block mTOR in its complex mTORC1 (27), abolished the detection of pS6-positive cells in the primary tumor site and invaded lymph nodes after its administration to orthotopic tumor–bearing mice (Fig. 4C and D and Supplementary Fig. S3C and S3D), confirming that the accumulation of pS6 reflects the aberrant activity of mTOR in these tumoral lesions. Interestingly, rapamycin and RAD001 also reduced pAktS473 levels in the primary tongue lesions and their

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**Figure 2.** Local invasive growth and lymphangiogenesis of orthotopically implanted HNSCC cells into the tongue. A, H&E-stained tissue section of an HNSCC orthotopic tumor (delimited with dotted line) growing into the anterior half of a tongue 4 weeks postimplantation. UMSCC2 is a well-differentiated squamous cell carcinoma displaying keratinization (inset, black arrowhead) and granular differentiation (inset, white arrowhead). B, intraepithelial invasion by HNSCC cells. The invasive area is indicated by the white arrows. The black arrow points to the remaining normal epithelium. C, perineural infiltration. A group of HNSCC cells (black arrow) are seen growing surrounding a nerve structure (white arrow). This was a common finding in this orthotopic HNSCC model. D, subepithelial lymphatic permeation by HNSCC cells. The carcinoma tends to grow inside the lymphatic vessels; this finding was confirmed by immunohistochemistry (below). E, immunohistochemical identification of vascular (CD31) and LYVE-1 cells was conducted and the results quantified in control mice and the HNSCC orthotopic tumors. No blood or lymphatic vessels are observed in normal epithelium. Normal mucosa included the epithelium and subepithelial area between the epithelium and the muscle; muscle refers to the normal skeletal muscle of the tongue, and tumor to the malignant epithelial area and its stroma.
metastases, suggesting that these rapalogs can also reduce mTORC2 activity in HNSCC, likely indirectly, as observed after prolonged treatment with rapamycin of cultured cells (31).

These observations prompted us to explore the consequences of treating mice harboring HNSCC tumors with rapamycin and RAD001. Treatment was initiated approximately 10 days after tumor implantation into the tongue when primary tumors were visible in all mice. As shown in Fig. 5A–D and Supplementary Fig. S4A–S4D, the impact of rapamycin treatment was remarkable. Weekly tongue evaluation revealed a significant tumor growth inhibition caused by rapamycin and RAD001 administration (Fig. 5A and Supplementary Fig. S4A). Typical examples of tumor-bearing mice treated with vehicle control, rapamycin, and RAD001 for approximately 2 to 3 weeks are depicted (Fig. 5B and Supplementary Fig. S4B). The orthotopic tongue HNSCC model enabled the
readily visualization of the tumoral lesions in the representative control and treated groups (Fig. 5C and Supplementary Fig. S4C). Quantification of the compromised tumoral area in each tongue showed a highly significant reduction of the affected tongue surface (Fig. 5D and Supplementary Fig. S4D).

The residual tumor in rapamycin- and RAD001-treated mice at the end of the observation period showed areas of squamous differentiation and fibrosis, in contrast to control-treated mice that showed active areas of cell growth (Fig. 6A–D and Supplementary Fig. S5A–S5D). Of interest, rapamycin and RAD001 did not affect the vascular microvessel density of the tumoral lesions and normal tissues in this orthotopic model (Fig. 6E and Supplementary Fig. S5E). However, they had a dramatic effect on the lymphatic system, as it prevented intratumoral lymphangiogenesis without perturbing the normal distribution of lymphatic vessels in the oral mucosa and muscle (Fig. 6E and Supplementary Fig. S5E). Aligned with this observation, rapamycin inhibits potently the proliferation of human lymphatic endothelial cells (Supplementary Fig. S6). On the other hand, the ability to monitor and quantitate lymph node invasion in this model system enabled us to explore whether the blockade of mTOR with rapamycin could impact HNSCC metastasis. As shown in Fig. 6F and Supplementary Fig. S5F, rapamycin and RAD001 treatment caused a remarkable decrease in the number of invaded lymph nodes, which was reflected in a significant increase in the overall survival of all rapamycin- and RAD001-treated animals (Fig. 6G and Supplementary Fig. S5G).
Discussion

Newly gained molecular understanding of HNSCC initiation and tumor evolution may soon afford the opportunity to delay or halt tumor progression. In this regard, among the multiple aberrant genetic, epigenetic, and signaling events known to occur in HNSCCs, the persistent activation of the Akt/mTOR pathway has emerged as potential drug target for HNSCC treatment. As supported by extensive preclinical investigation, the use of mTOR inhibitors, including rapamycin (sirolimus) and its analogues, CCI-779 (temserolimus), and RAD001 (everolimus), can dramatically reduce tumor burden and even recurrence in HNSCC xenografts and in chemically induced and genetically defined animal models recapitulating HNSCC initiation and progression (18–22). Furthermore, recent clinical evaluation of temserolimus as neoadjuvant prior to definitive treatment has revealed that all predicted biochemical targets for mTOR inhibitors in this tumor type are hit in the clinical setting, at clinically relevant doses and with limited side effects, resulting in cancer cell apoptosis and tumor shrinkage (32). We now show that activation of the mTOR pathway is a frequent event in human metastatic HNSCC lesions. Furthermore, by the use of an orthotopic model of HNSCC in which the local tumoral invasion and lymph node metastasis can be readily assessed, we now show that mTOR inhibition with rapamycin can reduce tumoral growth in the tongue, one of its most frequent sites. As the immune system plays an important role in tumor metastasis, the implantation of human HNSCC cells in immunodeficient mice may not reflect the clinical situation fully. While keeping this potential limitation in mind, this orthotopic animal model revealed that the treatment with rapamycin prevents the metastatic spread of the HNSCC lesions, thereby prolonging animal survival.

The blockade of mTOR in experimental and clinical HNSCC lesions leads to a rapid decrease in the phosphorylated state of S6 and 4EBP1 (18, 20, 32), 2 downstream targets of the mTOR complex 1 (mTORC1; ref. 27), which also serves as a biomarker.
for the validation of the biochemical activity of mTOR inhibitors in their target tissues. In HNSCCs, rapamycin also causes a rapid decrease in the phosphorylation of Akt in serine 473 (18–20), a target for mTORC2 (27), suggesting that, as shown in cultured cell systems (31), prolonged exposure to rapamycin and its analogues can reduce mTORC2 activity, likely by an indirect, yet unknown mechanism. Similarly, we have observed a rapid blockade of mTORC2 in the HNSCC orthotopic model system, as judged by decreased levels of pAktS473.

This effect could contribute to the antimetastatic activity of rapamycin, as mTORC2 is known to be involved in polarized cell migration in multiple cell types and even in model organisms (33, 34). Thus, the blockade of mTORC2 in HNSCCs might result in reduced migration of cancer cells toward chemoattractants often implicated in HNSCC metastasis, a possibility that is under current investigation.

Of interest, melanoma and HNSCCs are one of the few cancers in which intratumoral lymphangiogenesis is known
to occur (35). Aligned with these observations, although angiogenesis is a frequent event in HNSCC models, we noticed the formation of a remarkable network of intratumoral lymphatic vessels in the primary tumor site, which was only observed in the orthotopic HNSCC system but not when tumors were implanted in other anatomic locations. The release of multiple lymphangiogenic growth factors by HNSCCs and stromal cells within the tumoral microenvironment in the tongue may account for this remarkable prolymphangiogenic activity of orthotopically implanted HNSCC cells and their metastatic potential (36–41), an issue that warrants further investigation. We also observed the growth of invading HNSCC cells within the lymphatic vessels, together suggesting that HNSCC cancer cells can promote the growth and recruitment of lymphatic endothelial cells or their progenitors and support their survival within the tumor microenvironment. This was nearly completely prevented by rapamycin and RAD001 treatment, supporting an antilymphangiogenic function of mTOR inhibitors when administered to mice bearing tumors in their natural microenvironment. This effect likely involves the impact of these rapalogs on mTOR function in the tumor cells and/or in the lymphatic endothelial cells, hence preventing lymphangiogenic signaling. While these possibilities are under investigation, we can conclude that our findings support a unique antilymphangiogenic function of mTOR inhibitors, which could have multiple beneficial clinical implications. Indeed, while further work may be required to define precisely how mTOR inhibitors act in HNSCC, the emerging information suggests that rapamycin may exert its antitumoral activity at multiple steps, reducing the growth and size of the primary tumor, preventing the formation of intratumoral lymphatic vessels, and likely reducing the migratory activity of HNSCC cells toward the lymph nodes, thus preventing the locoregional metastatic spread of primary HNSCC lesions.

Among the factors influencing patient outcome, the presence of lymph node metastasis at the time of diagnosis represents the most important factor predicting a poor prognosis (5, 9–12). Unfortunately, tumor recurrence in successfully treated patients with HNSCC is a frequent event, often accompanied with metastatic disease even in prior lymph node–negative cases (42). Indeed, patients with HNSCC often succumb to metastatic disease, compromising both quality of life and overall life expectancy. Unfortunately, there are still limited therapeutic options to prevent disease progression and locoregional and distant HNSCC spread. In this regard, the emerging preclinical and clinical information about the promising beneficial effects of mTOR inhibitors in HNSCCs and our present findings can now be exploited to prevent HNSCC recurrence and metastasis. Specifically, we can envision that the present study and prior reports may provide a rationale for the future clinical evaluation of rapamycin and its analogues in an adjuvant setting, as part of a molecular-targeted strategy for metastasis prevention after definitive treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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