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Expression TGM2 and BNIP3 have prognostic significance in laryngeal cancer patients receiving surgery and postoperative radiotherapy: A retrospective study

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Abstract

Background: This study was designed to determine the pattern and correlation between expression of the HIF-1 α transcriptional targets TGM2 and BNIP3 in laryngeal cancer, and investigate the association of BNIP3 and TGM2 with clinical outcome in laryngeal squamous cell carcinoma (SCC) patients receiving postoperative radiotherapy.

Methods: Immunostaining with antibodies specific to BNIP3 and TGM2 was performed in formalin-fixed, paraffin-embedded specimens from 148 laryngeal SCC patients. BNIP3 and TGM2 expression was scored as high or low, based on the number of tumor cells stained and the staining intensity. All patients received postoperative radiotherapy. Patient follow up and clinicopathological data were compared using the Chi-squared test, univariate and multivariate analyses, and survival curves were generated using the Kaplan-Meier method and log-rank test.

Results: The 3, 5 and 10-year overall survival rates (OS) for all patients were 77.7%, 71.6%, 56.4%, respectively. Primary tumor site, T stage, overall stage, lymph-node metastasis, BNIP3 expression and TGM2 expression were significant prognostic factors for OS in univariate analysis. Negative cervical lymph nodes, high BNIP3 expression and low TGM2 expression were independent prognostic factors of improved OS in multivariate analysis. BNIP3 expression correlates with TGM2 expression in laryngeal SCC ($P = 0.012$).

Conclusions: This study indicates that lymph-node metastasis, BNIP3 expression and TGM2 expression are independent prognostic factors in laryngeal SCC patients receiving postoperative radiotherapy. Further studies are required to investigate how BNIP3 and/or TGM2 influence the prognosis of laryngeal SCC patients treated with postoperative radiotherapy, and to determine how TGM2 and BNIP3 expression are regulated.

Keywords: TGM2, BNIP3, Postoperative radiotherapy, Laryngeal cancer, Prognosis

Background

In the United States, laryngeal cancer accounted for approximately 0.85% of new cancer diagnoses and 0.65% of all cancer deaths in 2008 [1]. Postoperative radiotherapy (PRT) is widely advocated for squamous cell carcinoma (SCC) of the head and neck patients with a high risk of recurrence after surgical resection.

The most important factor in the prognostic evaluation of head and neck squamous cell carcinoma (HNSCC) is the tumor node metastasis (TNM) staging system, of which nodal stage is the most relevant factor. However, the outcome of patients with the same TNM stage can vary, which has led to a concerted effort to define additional TNM subcategories with a similar prognosis, and recent research has focused on the identification of molecular and biologic prognostic factors, regardless of TNM staging.

The hypoxic fraction of human cancers is resistant to radiation therapy due to reduced generation of oxygen

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radicals. The transcription factor hypoxia-inducible factor-1 (HIF-1 α) upregulates expression of a variety of target genes under hypoxic conditions, and plays a major role in determining tumor radiosensitivity. Therefore, HIF-1 α and HIF-1 α target genes represent potential therapeutic targets to influence the effect of hypoxia on tumor radiosensitivity.

BNIP3, a hypoxia-inducible pro-apoptotic gene belonging to the BCL2 family, was originally identified as an adenovirus E1B19-kDa protein-interacting gene. In normal tissues, BNIP3 expression is upregulated in hypoxic conditions by hypoxia inducible factor HIF-1 α and can lead to cell death [2-4]. In tumors, *BNIP3* is silenced via epigenetic mechanisms, such as promoter hypermethylation and histone deacetylation [5]. Down-regulation of BNIP3 results in the failure of tumor cells to undergo cell death, and is associated with chemoresistance and poorer survival [6,7].

The transglutaminase 2 (TGM2) family catalyze formation of an amide bond between the carboxamide groups of peptide-bound glutamine residues and primary amino groups in various compounds [8]. One member of the TGM2 family, TGase 2 (TGM2) can enhance the survival of hypoxic cells and has been identified as a HIF-1 α transcriptional target. Hypoxia upregulates *TGM2* expression via a HIF-1 α dependent pathway and concurrently activates intracellular TGM2 activity [9]. Increased expression of TGM2 is associated with drug resistance in cancer [10], due to activation of nuclear factor-kB (NF-kB) via cross-linking and polymerization of free I-kB by TGM2 [11].

Although BNIP3 and TGM2 are associated with drug resistance and provide valuable prognostic markers in a variety of cancers [6,7,10], the expression and significance of BNIP3 and TGM2 have not been investigated in patients with laryngeal SCC receiving postoperative radiotherapy. Therefore, we examined the pattern and correlation between TGM2 and BNIP3 expression to determine their association with clinical factors and outcome in patients with SCC of the larynx.

The results of this study indicate that, in addition to lymph node involvement, the expression levels of BNIP3 and TGM2 are novel independent predictive factors for survival in laryngeal SCC patients receiving postoperative radiotherapy.

Methods

Patients and tissue samples

This study was approved by the Institutional Review Board and Human Ethics Committee of Sun Yet-sen University Cancer Center. A total of 148 patients with histologically confirmed SCC of the larynx treated from 1997 to 2003 at the Sun Yet-sen University Cancer Center were included. Relevant clinical pathologic features

(Table 1) were obtained from the medical files and/or by telephone interviews with the patient or their relatives. Tumor types and histological grade classifications were designated according to World Health Organization classification of Tumors: Pathology and Genetics of Head and Neck Tumors [12].

Table 1 Expression of TGM2 and BNIP3 and their relationship with clinicopathological characteristics in laryngeal squamous cell carcinoma patients

| Features | No. of patients | TGM2 | | P | BNIP3 | | P |
|--------------------------------|-----------------|------|-----|--------------------|-------|-----|--------------------|
| | | High | Low | | High | Low | |
| Gender | | | | | | | |
| Female | 3(2.0%) | 0 | 3 | 0.283 ^a | 1 | 2 | 0.194 ^b |
| Male | 145(98.0%) | 70 | 75 | | 105 | 40 | |
| Age (years) ^c | | | | | | | |
| < 60 | 72(48.6%) | 35 | 37 | 0.755 | 52 | 20 | 0.875 |
| ≥60 | 76(51.4%) | 35 | 41 | | 54 | 22 | |
| Histological grade | | | | | | | |
| Well | 70(47.3%) | 37 | 33 | 0.184 | 50 | 20 | 0.933 |
| Moderately | 58(39.2%) | 22 | 36 | | 41 | 17 | |
| Poorly | 20(13.5%) | 11 | 9 | | 15 | 5 | |
| Smoking index ^d | | | | | | | |
| < 600 | 67(45.3%) | 36 | 31 | 0.154 | 52 | 15 | 0.651 |
| ≥600 | 81(54.7%) | 34 | 47 | | 54 | 27 | |
| Alcohol consumption | | | | | | | |
| No | 88(59.5%) | 40 | 48 | 0.587 | 56 | 32 | 0.009** |
| Yes | 60(40.5%) | 30 | 30 | | 50 | 10 | |
| Primary Site | | | | | | | |
| Glottic | 104(70.3%) | 50 | 54 | 0.433 | 74 | 30 | 0.791 |
| Supraglottic | 39(26.3%) | 19 | 20 | | 29 | 10 | |
| Subglottic | 5(3.4%) | 1 | 4 | | 3 | 2 | |
| T Stage | | | | | | | |
| 1-2 | 101(68.2%) | 47 | 54 | 0.785 | 71 | 30 | 0.600 |
| 3-4 | 47(31.8%) | 23 | 24 | | 35 | 12 | |
| Cervical lymph node metastasis | | | | | | | |
| Positive | 19(12.8%) | 9 | 10 | 0.995 | 17 | 2 | 0.064 |
| Negative | 129(87.2%) | 61 | 68 | | 89 | 40 | |
| TNM Stage | | | | | | | |
| I-II | 96(64.9%) | 46 | 50 | 0.838 | 67 | 29 | 0.502 |
| III-IV | 52(35.1%) | 24 | 28 | | 39 | 13 | |
| Total radiation dose | | | | | | | |
| < 60 Gy | 61(41.2%) | 24 | 37 | 0.105 | 41 | 20 | 0.319 |
| ≥60 Gy | 87(58.8%) | 46 | 41 | | 65 | 22 | |
| Margin | | | | | | | |
| Negative | 68(45.9%) | 38 | 30 | 0.054 | 50 | 18 | 0.635 |
| Positive | 80(54.1%) | 32 | 48 | | 56 | 24 | |
| BNIP3 | | | | | | | |
| High | 106(71.6%) | 57 | 49 | 0.012* | | | |
| Low | 42(28.4%) | 13 | 29 | | | | |

^a Continuity correction. ^bFisher's exact test. ^c Patients were divided according to the median age. ^d Smoking index is defined as the number of cigarettes used per day \times the total smoking time (years). * $P < 0.05$, ** $P < 0.01$

Surgery

All patients underwent a major surgical intervention at Sun Yet-sen University Cancer Center; patients irradiated after excisional biopsies were not included in the study. In total, 116/148 (78.4%) of the laryngeal SCC patients were treated with partial laryngectomy and 32/148 (21.6%) were treated with total laryngectomy. Unilateral or bilateral neck node dissection was performed in 79 (53.4%) of the patients.

Radiotherapy

The indications for postoperative radiotherapy in our hospital at the time of the study included clinically or microscopically positive surgical margins, pathologically confirmed positive neck nodes and/or the advanced clinical stages of primary laryngeal cancer (T3, T4). Only patients in good general condition with no distant metastases were considered for PRT.

The total radiation dose in the clinical target volume area ranged from 36 to 82 Gy, with a median value of 60 Gy. Lower doses (< 50 Gy) were given to patients whose clinical condition demanded premature termination of the treatment (due to local progression during PRT, deteriorating performance status or intercurrent disease) or patients who refused to complete treatment. Higher doses (> 70 Gy) were given to patients with a clinically palpable mass after surgery, or patients who required an increased total dose due to long duration treatment gaps.

Immunohistochemistry (IHC) staining

A total of 148, routinely processed laryngeal squamous cell carcinoma paraffin-embedded samples were cut into 4 μ m sections, dried overnight at 56°C, deparaffinized, rehydrated, antigen retrieval was performed in citrate buffer at 92°C using a microwave oven and the sections were incubated in 3% H₂O₂ to block endogenous peroxidase activity. The sections were incubated in mouse monoclonal [CUB 7402] anti-TGM2 antibody (ab2386, ABCAM, dilution 1:100) or mouse monoclonal [Ana40] anti-BNIP3 antibody (ab10433, ABCAM, dilution 1:100) overnight at 4°C in a humidified chamber, staining was visualized using an avidin-biotin technique and DAB staining, followed by hematoxylin nuclear counterstaining. Negative controls were prepared by omitting the primary antibody. Histological and IHC evaluation were performed independently by two pathologists while blinded to the clinicopathological outcomes of the patients. Slides with indeterminate evaluation were re-evaluated, and a consensus was reached. Briefly, each slide was examined in its entirety under a light microscope. Pilot studies indicated BNIP3 staining was both nuclear and cytoplasmic (Figure 1); therefore, the intensity of nuclear and cytoplasmic staining was defined as

no staining (0), weak staining (1), moderate staining (2) or strong staining (3), and the percentage of cells stained was scored as 0-10% (1), 10-50% (2), 51-80% (3) or 81-100% (4). Tumors were considered highly positive if they demonstrated moderate or strong staining (2 or 3) in greater than 50% of cells (3 or 4). TGM2 expression was scored using the same intensity, percentage scores and positive criteria as BNIP3, as previously reported [13].

Statistical methods

Statistical analysis was performed using SPSS 13.0 for Windows. The χ^2 test was used to evaluate categorical variables. Associations between clinicopathological features and TGM2 or BNIP3 immunohistochemical expression were analyzed using the Pearson chi-square test of independence. Multivariate survival analyses were performed using the Cox regression model. Overall survival (OS) was measured from the onset of treatment to the date of death or survival status at the last date of follow-up. OS probabilities were estimated using the Kaplan-Meier method and significant differences were assessed using the log-rank test. *P* values < 0.05 were considered statistically significant, and *P* values < 0.01 were considered strongly statistically significant.

Results

Clinicopathological features

In total, 148 laryngeal SCC patients (145 males and 3 females) with a median age of 60 years (range: 23-85 years) were included in this study. Table 1 presents a summary of the gender, age, tumor site, tumor stage, histological grade, smoking index, alcohol consumption, total radiation dose and surgery margins of the patients. According to the 6th Edition of the International Union Against Cancer (UICC) TNM classification system, 26 patients were Stage I, 70 were Stage II, 28 were Stage III and 24 were Stage IV.

Patient outcomes after follow-up

The last follow-up date was June 29th, 2010, and no patients were lost during follow-up. The 3, 5 and 10 year overall survival rates (OS) for all patients were 77.7%, 71.6% and 56.4%, respectively (Figure 2). Sixty two cancer-related deaths were reported, with a median time to death of 35 months (range 1-108 months). There are 6 non-cancer-related deaths. The median time to non-cancer-related death is 30 months (range 18-78 months).

Immunohistochemical expression of TGM2 and BNIP3

Positive expression of BNIP3 was mainly localized in the nucleus, with some expression detected in the cytoplasm of laryngeal SCC tumor cells. Positive expression of

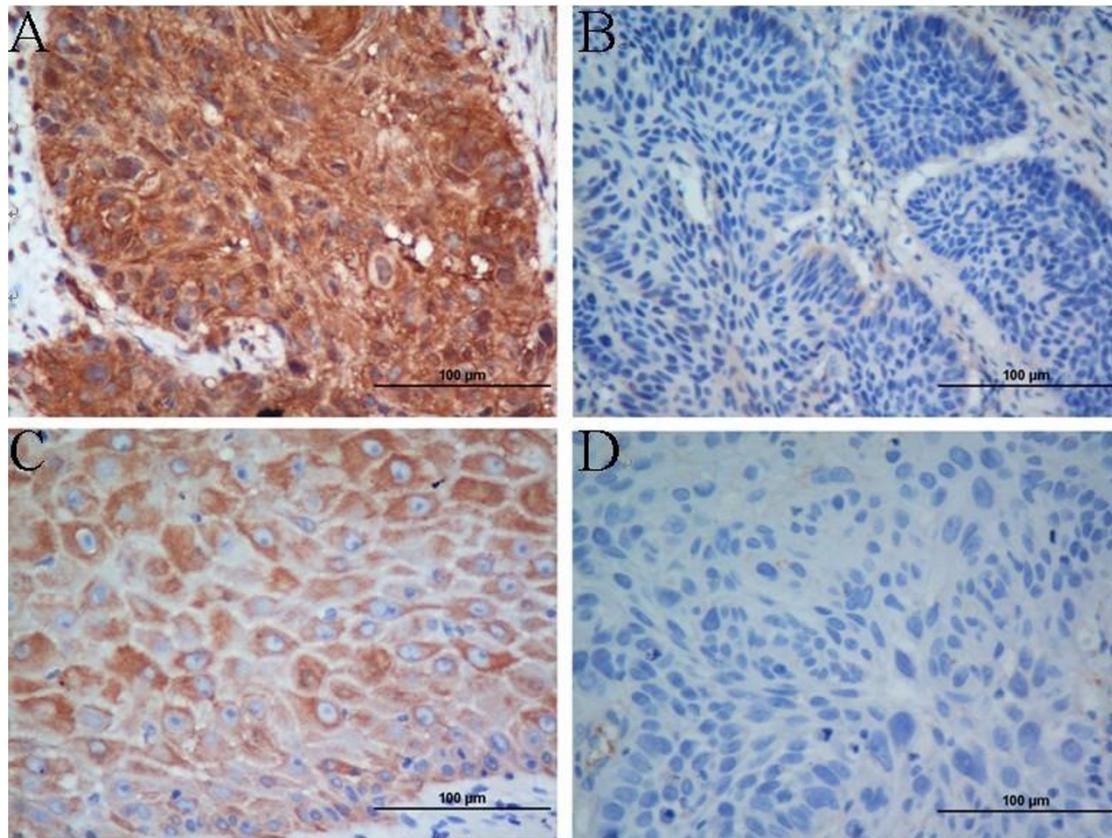


Figure 1 Immunohistochemical staining for TGM2 and BNIP3 in laryngeal squamous cell carcinoma. Positive expression of BNIP3 was mainly localized in the nucleus, but was also observed in the cytoplasm of tumor cells. Positive expression of TGM2 was mainly localized in the cytoplasm of tumor cells, but was also observed in the nucleus. Representative images of (A) highly positive BNIP3 expression in tumor cells, (B) low BNIP3 expression in tumor cells, (C) highly positive TGM2 expression in tumor cells and (D) low TGM2 expression in tumor cells (SP × 400)

TGM2 was mainly localized in the cytoplasm of laryngeal SCC tumor cells, with some expression detected in the nucleus (Figure 1). The immunostaining scores are listed in the Table 1. TGM2 was highly positive in 47.3% (70/148) of the tumors, and BNIP3 was highly positive in 71.6% (106/148) of the tumors. Furthermore, highly positive BNIP3 expression was correlated with highly positive TGM2 expression ($P = 0.012$) and alcohol consumption ($P = 0.009$; Table 1).

BNIP and TGM2 are associated with patient prognosis

Univariate analyses indicated a significant association between OS and primary tumor site, T stage, cervical lymph node metastasis, TNM stage, TGM2 expression and BNIP3 expression in laryngeal SCC (Table 2, Figure 2). Patients with both high BNIP3 expression and low TGM2 expression had a significantly better overall survival, compared to patients with low BNIP3 expression and high TGM2 expression, respectively ($P < 0.001$). The 3, 5 and 10-year overall survival rates (OS) of patients who

expressed high levels of BNIP3 and low levels of TGM2 were 87.8%, 85.7% and 76.9%, respectively.

Multivariate analysis

Multivariate survival analysis was performed using the Cox regression model to calculate the odds ratio and 95% confidence intervals for each clinicopathological variable. The model was simplified in a stepwise fashion by removing variables with a P value ≥ 0.05 . The analysis revealed that TGM2 expression status, BNIP3 expression status and cervical lymph node metastasis were statistically significant independent predictive factors for OS in laryngeal SCC patients (Table 3).

Discussion

In this study we evaluated the significance of various prognostic factors in patients with SCC of the larynx treated with primary surgery and PRT. No patients received any other form of adjuvant systemic treatment. The overall survival (OS) of the patients was

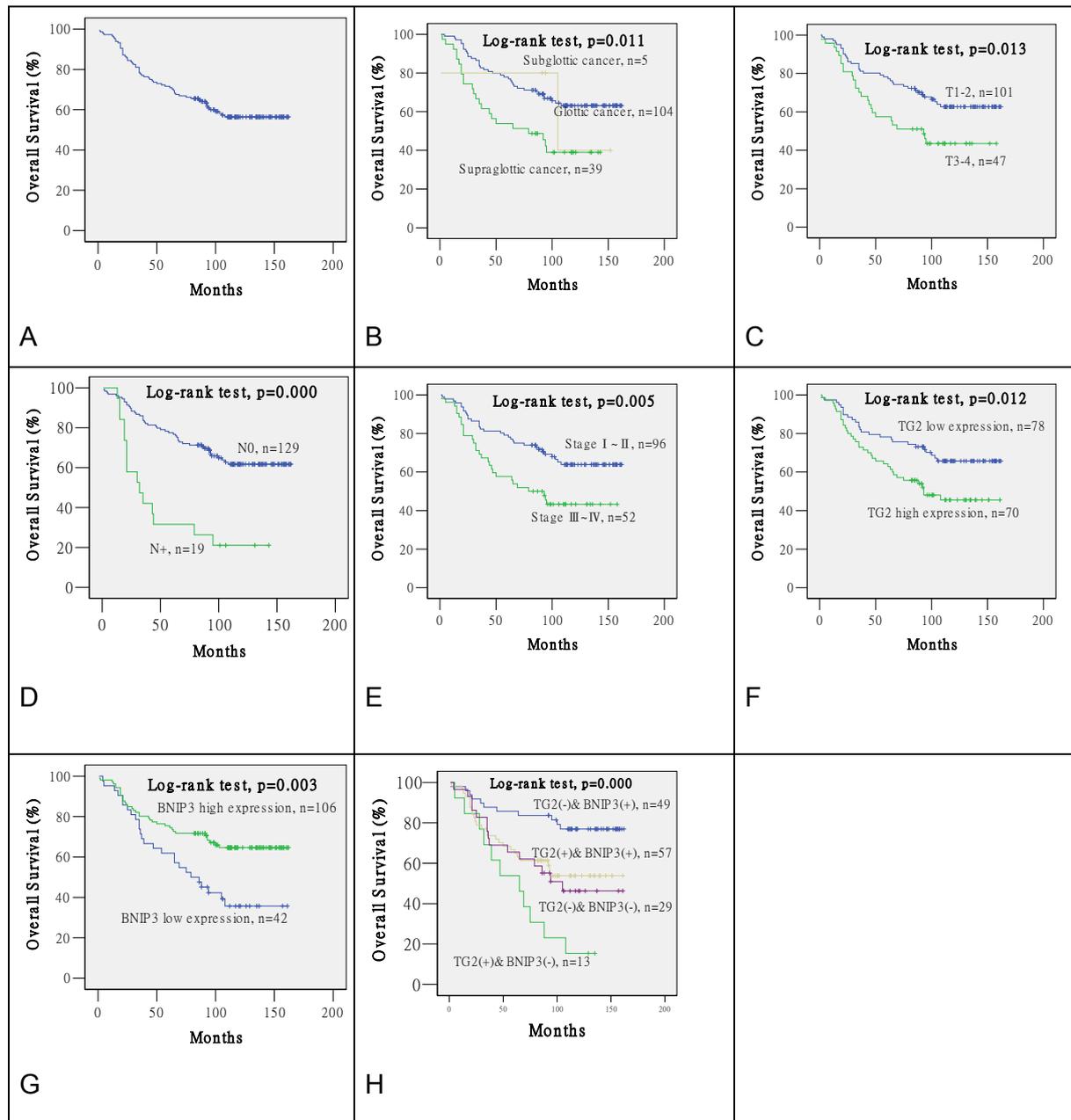


Figure 2 Survival curves for laryngeal squamous cell carcinoma patients. (A) Overall survival (OS) for all patients. (B) OS in patients with different primary tumor sites. (C) OS in patients with different T stages. (D) OS in patients with and without cervical lymph node metastasis. (E) OS in patients with different UICC stages. (F) OS in patients with high and low TGM2 expression. (G) OS in patients with high and low BNIP3 expression. (H) OS in patients with high and low BNIP3 and TGM2 expression.

significantly related to the UICC stage, in agreement with previous reports that the initial classification (T stage, N stage and total stage) are significant prognostic factors for survival in laryngeal SCC [14-17]. In this study, univariate analysis also indicated that tumor sites in the supraglottis were correlated with a significantly poorer prognosis.

It has been reported that the number, site and volume of metastatic lymph nodes can negatively influence the prognosis in laryngeal carcinoma [18,19], and these results were confirmed by our study, as univariate analysis indicated that the presence of metastatic lymph nodes ($P < 0.001$) had a negative prognostic value. The relationship between nodal involvement and local

Table 2 The relationship of clinicopathological variables and immunohistochemical features with overall survival (OS) in laryngeal squamous cell carcinoma patients

| Feature | No. of patients | Overall survival (%) | | | χ^2 | P ^a |
|--------------------------------|-----------------|----------------------|------|------|----------|----------------|
| | | 3 y | 5 y | 10 y | | |
| Gender | | | | | 1.539 | 0.215 |
| Female | 3(2.0%) | 100 | 100 | 100 | | |
| Male | 145(98.0%) | 77.2 | 71.0 | 55.6 | | |
| Age (years) ^b | | | | | 0.019 | 0.889 |
| < 60 | 72(48.6%) | 80.6 | 72.2 | 54.9 | | |
| ≥60 | 76(51.4%) | 75 | 71.1 | 57.6 | | |
| Histological grade | | | | | 2.976 | 0.226 |
| Well | 70(47.3%) | 85.7 | 81.4 | 60.8 | | |
| Moderately | 58(39.2%) | 69.0 | 62.1 | 51.2 | | |
| Poorly | 20(13.5%) | 75.0 | 65.0 | 55.0 | | |
| Smoking index ^c | | | | | 3.745 | 0.053 |
| < 600 | 67(45.3%) | 83.6 | 77.6 | 66.2 | | |
| ≥600 | 81(54.7%) | 72.8 | 66.7 | 48.4 | | |
| Alcohol consumption | | | | | 0.033 | 0.856 |
| No | 88(59.5%) | 79.5 | 71.6 | 56.8 | | |
| Yes | 60(40.5%) | 75.0 | 71.7 | 55.6 | | |
| Primary Site | | | | | 8.974 | 0.011* |
| Glottic | 104(70.3%) | 82.7 | 77.9 | 63.1 | | |
| Supraglottic | 39(26.3%) | 64.1 | 53.8 | 39.0 | | |
| Subglottic | 5(3.4%) | 80.0 | 80.0 | 40.0 | | |
| T Stage | | | | | 6.201 | 0.013* |
| 1-2 | 101(68.2%) | 81.2 | 78.2 | 62.6 | | |
| 3-4 | 47(31.8%) | 70.2 | 57.4 | 43.5 | | |
| Cervical lymph node metastasis | | | | | 21.263 | < 0.001** |
| Negative | 129(87.2%) | 82.9 | 77.5 | 61.7 | | |
| Positive | 19(12.8%) | 42.1 | 31.6 | 21.1 | | |
| TNM Stage | | | | | 7.736 | 0.005** |
| I-II | 96(64.9%) | 82.3 | 79.6 | 63.8 | | |
| III-IV | 52(35.1%) | 69.2 | 57.7 | 43.3 | | |
| Total radiation dose | | | | | 1.671 | 0.196 |
| < 60 Gy | 61(41.2%) | 78.7 | 75.4 | 62.9 | | |
| ≥60 Gy | 87(58.8%) | 77.0 | 69.0 | 51.7 | | |
| Margin | | | | | 0.395 | 0.530 |
| Negative | 68(45.9%) | 82.4 | 76.5 | 57.9 | | |
| Positive | 80(54.1%) | 73.8 | 67.5 | 55.0 | | |
| TGM2 | | | | | 6.308 | 0.012* |
| High | 70(47.3%) | 72.9 | 64.3 | 45.5 | | |
| Low | 78(52.7%) | 82.1 | 78.2 | 65.7 | | |
| BNIP3 | | | | | 8.586 | 0.003** |
| High | 106(71.6%) | 79.2 | 75.5 | 64.4 | | |
| Low | 42(28.4%) | 71.4 | 61.9 | 35.7 | | |
| TGM2 & BNIP3 | | | | | 19.021 | < 0.001** |
| TGM2 (-) & BNIP3 (+) | 49(33.1%) | 87.8 | 85.7 | 76.9 | | |
| TGM2 (+) & BNIP3 (-) | 13(8.8%) | 69.2 | 53.8 | 15.4 | | |

Table 2 The relationship of clinicopathological variables and immunohistochemical features with overall survival (OS) in laryngeal squamous cell carcinoma patients (Continued)

| | | | | |
|----------------------|-----------|------|------|------|
| TGM2 (+) & BNIP3 (+) | 57(38.5%) | 73.7 | 66.7 | 53.8 |
| TGM2 (-) & BNIP3 (-) | 29(19.6%) | 72.4 | 65.5 | 46.3 |

^a Log-rank test. ^b Patients were divided into two groups according to the median age. ^c Smoking index is defined as the number of cigarettes used per day × the total smoking time (years). *P < 0.05, **P < 0.01

control has been examined in several patient series. van den Bogaert et al. and Wall et al. [20,21] reported 5 year local control rates of 70% and 79% in patients without positive node involvement, compared to 49% and 57% in patients with clinically positive nodes. Three large multicenter studies of laryngeal SCC patients treated with PRT have reported similar recurrence rates [22-24], indicating that cervical lymph node status is the most important tumor-related prognostic factor in head and neck cancer patients. Additionally, the incidence of local recurrence and risk of distant metastases increase as the tumor burden in the neck increases [25,16].

To our knowledge, no previous studies have evaluated the prognostic value of TGM2 and BNIP3 in head-and-neck cancer in patients treated with PRT. In this study, univariate analyses and multivariate analysis indicated that high expression of TGM2 and/or low expression of BNIP3 are associated with poorer overall survival in laryngeal SCC patients receiving PRT.

Several factors may explain the association between high expression of TGM2 and poorer OS. TGM2 expression can promote cell surface interaction with fibronectin and protect breast cancer cells from apoptosis [26]. Intense expression at the stromal-epithelial interface suggests that TGM2 plays a role in cell adhesion, cell migration, invasiveness and metastasis [27], and Dardik et al. reported that stromal expression of TGM2 in the endothelium of newly formed blood vessels may lead to tumor growth and metastasis [28].

Table 3 Multivariate analysis of the effect of clinicopathological factors on overall survival in laryngeal squamous cell carcinoma patients

| Variables | Overall survival | | |
|--------------------------------|------------------|-------------|-----------|
| | Odds ratio | 95% CI | P values |
| Cervical lymph node metastasis | 3.708 | 1.952-7.041 | < 0.001** |
| TGM2 | 2.899 | 1.669-5.036 | < 0.001** |
| BNIP3 | 0.324 | 0.188-0.559 | < 0.001** |
| Primary Site | 1.460 | 0.934-2.283 | 0.097 |
| T Stage | 1.485 | 0.871-2.534 | 0.147 |

*P < 0.05, **P < 0.01

Additionally, TGM2 can activate NF- κ B by crosslinking and polymerization, or by destabilizing the association of the p65/p50 (NF- κ B) complex with I κ B [27]. Aberrant activation of NF- κ B complexes can contribute to tumorigenesis by regulating expression of genes which promote cancer cell growth and survival. Constitutive expression of NF- κ B has also been implicated in tumor drug resistance. Recent experimental studies by Dae-Seok Kim et al. demonstrated that inhibition of TGase 2 using siRNA, cystamine, glucosamine or R2 peptides promotes cell death in drug-resistant cancer cells via NF- κ B inactivation [29]; However, Barnes et al. [30] suggested that reduced TGM2 activity is associated with tumor growth and metastasis, while Fesus et al. reported that increased activity of TGM2 in cells undergoing apoptosis indicates a pro-apoptotic function [31]. Collectively, these studies indicate that the role of TGM2 may depend on the type of cancer, the cell type and cancer stage; and this study demonstrates that high positive expression of TGM2 is associated with poorer overall survival in laryngeal SCC patients receiving surgery and postoperative radiotherapy.

Little is known about how TGM2 expression may affect the treatment efficacy of radiotherapy. Yuan et al. reported that the small molecule TGM2 inhibitor KCC009, which inhibits the ability of TGM2 to bind fibronectin and prevents the disposition of linear fibronectin strands in the ECM, promotes apoptosis and enhances radiosensitivity in cultured IOMM-Lee meningioma cells and meningioma tumor explants [32]. Mian et al. discovered that overexpression of TGM2 or a cross-linking defective TGM2 mutant (C277S) in malignant hamster fibrosarcoma cells (MetB) lead to delayed S-phase to G2/M progression [33]. As the G2/M-phase is the most sensitive phase to radiation, this observation may explain the association between overexpression of TGM2 and adverse outcome in patients receiving radiotherapy. Though this evidence strongly suggests a role for TGM2 in cancer, the exact molecular mechanism which by high levels of TGM2 expression lead to an adverse prognosis in laryngeal SCC is not known and requires further research.

BNIP3 is strongly upregulated in response to hypoxia. Generally, overexpression of BNIP3 induces necrotic-like cell death, autophagy or apoptotic cell death. However, it has been suggested that the ability of BNIP3 to induce cell death is blocked in cancer cells [34]. BNIP3 is downregulated in pancreatic ductal adenocarcinoma and correlates with reduced patient survival [5,6]. BNIP3 is also downregulated in oxaliplatin-resistant colon cancer cells [7] and knockdown of BNIP3 using siRNA or S100A4 results in resistance to fluorouracil (5-FU) and/or gemcitabine in pancreatic ductal adenocarcinoma

cells [6], indicating that BNIP3 is an important regulator during drug-induced cell death in cancer cells.

This study indicates that reduced expression of BNIP3 is associated with poorer overall survival in laryngeal SCC patients receiving PRT. The ability of BNIP3 to affect the efficacy of radiotherapy is poorly characterized. Kennedy et al. observed that overexpression of the apoptosis-related gene BNIP3 could affect the sensitivity of CRC cells to combined paclitaxel, radiation, and 5-FU therapy [35], and the molecular mechanism by which BNIP3 expression affects the prognosis of patients with laryngeal SCC requires further study.

In this study we observed that BNIP3 expression was correlated with TGM2 expression in laryngeal SCC ($P = 0.012$). TGM2 activates NF- κ B, via crosslinking and polymerization or destabilizing the association of the p65/p50 (NF- κ B) complex with I κ B [27]. Therefore, we hypothesize that increased expression of TGM2 leads to down-regulation of BNIP3 because BNIP3 expression can be repressed through the transcription factor nuclear factor kappa B (NF- κ B) binding to the bnip3 promoter [36].

The results of this study should be interpreted with caution as this is a retrospective study of a relatively small number of samples. Use of alternative methodologies such as microdissection and in-situ hybridization and/or RT-PCR are required to confirm these results at the RNA level.

Conclusions

The results of this study indicate that the expression levels of BNIP3 and TGM2 are novel predictive factors of survival in laryngeal SCC patients treated with surgery and postoperative radiotherapy, and further study is required to determine the molecular mechanisms by which BNIP3 and TGM2 influence the prognosis of these patients. Additionally, the mechanism regulating the correlation between TGM2 and BNIP3 expression in laryngeal SCC requires further investigation.

Abbreviations

PRT: Postoperative radiotherapy; SCC: Squamous cell carcinoma; HIF-1: Hypoxia-inducible factor-1; TGM2: Transglutaminase 2; NF- κ B: Nuclear factor- κ B; IHC: Immunohistochemistry; UICC: International Union Against Cancer; OS: Overall survival.

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Authors' contributions

TJ, HXL, LBG, HL, RS, WKC, and QLL carried out the cases collection, TJ and HL carried out the immunohistochemical staining work, TJ, HL, XYZ and WHH analyzed results. WHH conceived of the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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