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MAIN ARTICLE

Does HMGB1 predict occult neck lymph node metastasis in early tongue carcinoma? A case–control study of 26 patients

H HANAKAWA[1], Y ORITA[1], Y SATO[2], M TAKEUCHI[2], S TAKAO[3], K OHNO[2], T KOHNO[1], N IWAKI[2], H MARUNAKA[4], R TAMAMURA[5], M NISHIBORI[6], H NAGATSUKA[5], K NISHIZAKI[1], T YOSHINO[2]

Departments of [1]Otolaryngology Head and Neck Surgery, [2]Pathology, [3]Epidemiology, [5]Oral Pathology and Medicine, and [6]Pharmacology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, and [4]Department of Otolaryngology Head and Neck Surgery, Okayama Medical Center, Japan

Abstract

Objective: This study examined whether the occurrence of late neck metastasis in early tongue squamous cell carcinoma can be predicted by evaluating HMGB1 (high mobility group box 1) expression in the primary lesion.

Methods: A case–control study was conducted. The cases comprised 10 patients with late neck metastasis. The controls consisted of 16 patients without recurrence. All were examined immunohistochemically for HMGB1 protein expression. The odds ratio for late neck metastasis in relation to HMGB1 was estimated.

Results: Results for HMGB1 were dichotomised into positive staining scores (score, 5–7) and negative scores (0–4). Six cases (60 per cent) and four controls (25 per cent) were HMGB1-positive. Although no significant result was seen, compared with HMGB1-negative patients the odds ratio for late neck metastasis in HMGB1-positive patients was 3.8 (95 per cent confidence interval, 0.6–26.5) after adjusting for other factors.

Conclusion: In the present study, immunohistochemical study of HMGB1 in early tongue squamous cell carcinoma did not appear to be very useful for predicting occult neck metastasis. Further study is necessary to clarify the relationship between HMGB1 expression and late neck metastasis in early tongue squamous cell carcinoma.

Key words: HMGB1; Tongue; Carcinoma; Metastasis

Introduction

The clinical course of early tongue carcinoma is difficult to predict using established risk factors. We sometimes encounter cases with a poor prognosis among patients who initially show a very superficial pathologically staged primary tumour (T_1), without clinical evidence of metastatic disease. The presence or absence of occult neck metastasis is one of the major prognostic factors in node-negative (N_0) tongue carcinoma.[1] The prevalence of occult neck metastasis among patients with early tongue carcinoma is estimated to be about 20–30 per cent. Several studies have addressed the need for prophylactic neck dissection in patients with clinically staged N_0 early tongue carcinoma (tumour–node–metastasis (TNM) staging of $T_{1/2}N_0M_0$).[2]

In Japan, many facilities employ a wait-and-see strategy for patients with clinical N_0 tongue carcinoma after close pre-operative image diagnosis. This is in light of the burden on the patient, and the risk of surgical complications such as palsy of the mandibular branch of the facial nerve and shoulder dysfunction.[3] As the Japanese medical insurance system allows all patients easy access to hospitals and frequent, careful follow up, immediate neck dissection can be performed shortly after neck metastasis is detected. However, the survival rate of patients with late metastasis is not high.[1,4] Depth of tumour invasion is the most widely accepted predictor of late metastasis,[1,5,6] but much room remains for evaluating prognosis more precisely.[7,8]

HMGB1 (high mobility group box 1), a chromatin-associated nuclear protein, is constitutively expressed in the nucleus of both cancer and normal cells. HMGB1 overexpression has been reported in a variety of human cancers, and has been identified as a general regulator of cell migration.[9] HMGB1 protein is claimed to affect cell invasion, tumour growth and metastasis by high-affinity binding to

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RAGE (receptor for advanced glycation end-products).[10] Previous studies have observed expression of HMGB1 in various stages of cancer, or have compared expression of HMGB1 in cancer with expression in normal tissues. The information that expression levels of HMGB1 are significantly higher in cancers than in normal tissues, or are significantly correlated with clinical stage or pathological grade of cancers, so far does not seem very useful for clinicians. However, HMGB1 might offer a target for treatment in cancer based on the inhibition of tumour cell invasion.[9,10]

The present study examined expression of HMGB1 in tongue squamous cell carcinoma (SCC), limited to the early stages (stage I or II according to the Union for International Cancer Control staging criteria), to demonstrate whether this factor can be used to predict the occurrence of late neck metastasis.

Materials and methods

Patients

Paraffin-embedded samples were obtained from 26 stage I or II primary tongue SCC patients (according to the Union for International Cancer Control staging criteria). Diagnoses in these 26 patients had previously been confirmed by physical examination, ultrasonography, computed tomography with or without ^{18}F-deoxyglucose positron emission tomography, and histological examination. If neck metastasis was suspected based on any of these preliminary examinations, ultrasound-guided fine needle aspiration cytology was performed to confirm a negative result. All specimens were obtained from patients who underwent partial glossectomy at Okayama University Hospital, Japan, without any other treatments, including prophylactic neck dissection, pre- or post-operative chemotherapy, or radiotherapy.

The cases comprised 10 patients who were treated between January 2005 and March 2012, and were found to have neck metastasis during follow up. The sites of late neck metastases were level 1 in two cases, level 2 in five, both levels 1 and 2 in two, and level 3 in one, respectively. The controls consisted of 16 patients who were treated between January 2005 and March 2008, and had been observed without recurrence for more than 5 years, thus being considered as having achieved complete cure. None of the 26 patients developed local recurrence or distant metastasis, and none were lost to follow up during the observation period.

Immunohistochemical staining

Serial sections (4 μm) were cut from each paraffin-embedded tissue block, and several sections were stained with haematoxylin and eosin. The sections were immunohistochemically stained with rat monoclonal anti-HMGB-1 antibody (10 μg/ml) using an automated Bond-Max stainer (Leica Biosystems, Melbourne, Australia). The anti-HMGB-1 antibody was provided by a pharmacologist.[11]

Staining evaluation

Immunohistochemically stained tissue sections were scored separately by three pathologists blinded to the clinical parameters. HMGB1 staining was mostly observed in the nuclei and cytoplasm of carcinoma cells and some fibroblasts. Staining intensity was scored as: 0 = negative, 1 = weak, 2 = medium or 3 = strong (Figure 1). The extent of staining to the entire carcinoma-involved area was scored as: 0 = 0 per cent, 1 = 1–25 per cent, 2 = 26–50 per cent, 3 = 51–75 per cent or 4 = 76–100 per cent. The sum of the intensity and extent scores was used as the final staining score (0–7) for HMGB1.[12] This grading system has previously been used for evaluation of HMGB1 expression in the immunohistochemically stained tissue sections of nasopharyngeal carcinoma.[12] After deciding on the scores independently, the three pathologists gathered to discuss appropriate scores and decide on the final scores. Patients were then categorised as HMGB1-positive (HMGB1 score = 5–7) or HMGB1-negative (HMBG1 score = 0–4).

Statistical analysis

We first assessed univariate associations between demographic characteristics and potential prognostic factors including HMGB1 expression and late neck metastasis. Crude odds ratios were then estimated. Logistic regression modelling was used to examine associations between HMGB1 and late neck metastasis, adjusting for other prognostic factors. Adjusted variables were selected for inclusion in the model on the bases of both the strength of the association and clinical importance. Odds ratios and 95 per cent confidence intervals (CIs) were calculated. Values of $p < 0.05$ (two-sided test) were considered statistically significant. All analyses were performed using SPSS version 21.0 J software (SPSS, Armonk, New York, USA) and STATA/SE 12.0 J software (Stata, College Station, Texas, USA).

Results

Of the 26 tongue SCC patients (Table I), 11 were female and 15 were male (57.7 per cent males), ranging in age from 29 to 87 years (median, 63 years; mean, 69 years). The primary site for all 26 patients was the edge of the tongue, with no cases showing the primary lesion at the centre or root of the tongue. All 26 patients underwent partial glossectomy without prophylactic neck dissection. Pathological study revealed that all the specimens were completely resected with sufficient margins. Of the 10 patients with late neck metastases, 8 survived and 2 died of the disease during the observation period. The interval between surgery for primary tongue carcinoma and presentation of neck metastases ranged from 3 to 18 months (mean, 8.5 months).

As summarised in Table II, no significant associations were seen between late neck metastases in early

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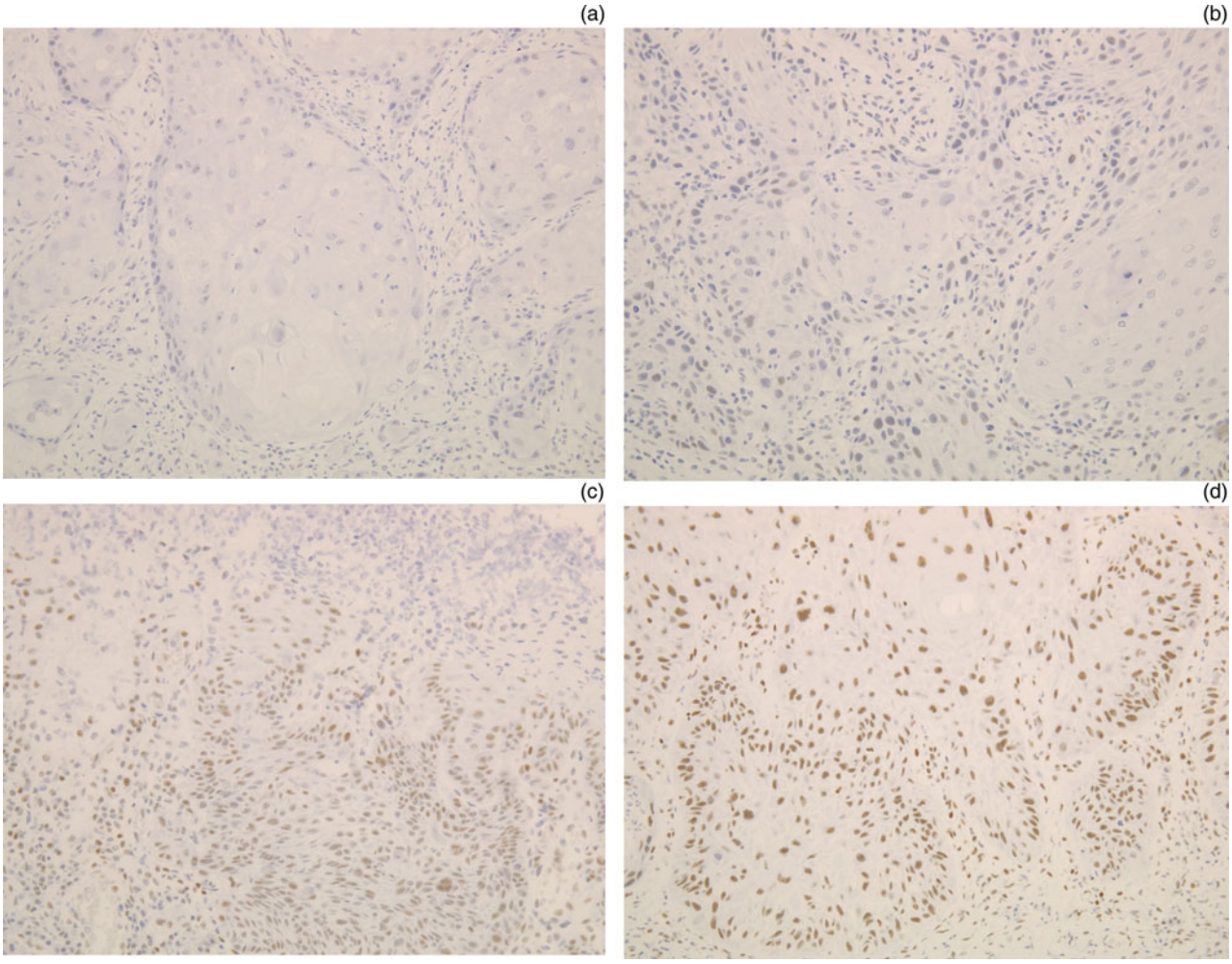

FIG. 1

HMGB1 expression in early tongue squamous cell carcinoma. Immunohistochemical staining intensity with HMGB1 antibody was scored as: 0 = negative (a), 1 = weak (b), 2 = moderate (c) or 3 = strong (d). (×200)

tongue carcinoma and sex, age, tumour classification (T_1 or T_2), pathological differentiation, or HMGB1 expression (positive *vs* negative). However, the point estimate of HMGB1 expression was 4.50 and indicated a relatively strong association.

Table III shows the adjusted odds ratios and 95 per cent CIs for HMGB1 and selected variables. As this study included only a small number of patients ($n = 26$), analysis of many of the prognostic factors by multivariate analyses in the same model might be imprecise and problematic. Based on both the strength of the associations and clinical importance, we selected sex as a demographic variable and tumour classification as a clinical characteristic for adjusting the relationship between HMGB1 and late neck metastasis. The adjusted odds ratio for positive HMGB1 (HMBG1 score of 5–7) was 3.82 (95 per cent CI, 0.55–26.50) compared with negative HMGB1 (HMBG1 score of 0–4).

Discussion

Our findings indicate that HMGB1 might not be useful as a predictive marker for late neck metastasis in early tongue SCC compared with previously established risk factors. Two possible explanations for our findings were considered: (1) HMGB1 is truly a prognostic marker, or (2) HMGB1 is a confounder that needs to be adjusted for when estimating associations with other potential predictors. As the point estimate showed a relatively strong association per se, HMGB1 might still be identified as a significant prognostic factor in a future study with more statistical power (i.e. many more patients). In addition, the odds ratios of sex and tumour stage T_2 were obviously increased after adjusting for HMGB1 in the same model. This might suggest HMGB1 as a possible confounder in determining relationships between other prognostic factors and late neck metastasis. The scoring system used for the evaluation of HMGB1 staining has been employed in previous studies.[12,13] In a study of HMGB1 expression in oesophageal SCC, the total staining score of HMGB1 was calculated by multiplying the intensity and extent scores.[14] We also used this calculation method to analyse the data, and attained almost the same results. It will be necessary to accumulate more cases to clarify the

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TABLE I
PATIENTS' DEMOGRAPHIC AND CLINICAL CHARACTERISTICS*

Patient number	Sex	Age† (years)	TNM	Primary site‡	Follow up (years)	Outcome	Interval** (months)
1	F	29	$T_1N_0M_0$	LE	7	A	–
2	F	32	$T_2N_0M_0$	LE	8	A	–
3	F	37	$T_1N_0M_0$	RE	5	A	–
4	F	51	$T_2N_0M_0$	LE	6	A	–
5	M	59	$T_1N_0M_0$	LE	5	A	–
6	M	59	$T_1N_0M_0$	LE	5	A	–
7	F	69	$T_1N_0M_0$	LE	7	A	–
8	M	72	$T_1N_0M_0$	LE	6	A	–
9	M	74	$T_1N_0M_0$	RE	5	A	–
10	M	75	$T_2N_0M_0$	LE	6	A	–
11	F	75	$T_1N_0M_0$	LE	5	A	–
12	M	76	$T_1N_0M_0$	RE	5	A	–
13	M	76	$T_1N_0M_0$	LE	5	A	–
14	F	78	$T_1N_0M_0$	LE	5	A	–
15	F	83	$T_2N_0M_0$	LE	5	A	–
16	F	87	$T_2N_0M_0$	LE	5	A	–
17	F	36	$T_2N_0M_0$	LE	1	A	3
18	M	47	$T_1N_0M_0$	RE	4	A	5
19	M	51	$T_1N_0M_0$	RE	2	A	15
20	M	52	$T_1N_0M_0$	RE	12	D	18
21	M	55	$T_2N_0M_0$	LE	8	A	10
22	M	66	$T_2N_0M_0$	RE	7	A	8
23	F	69	$T_2N_0M_0$	RE	3	A	3
24	M	69	$T_1N_0M_0$	LE	1	D	8
25	M	73	$T_2N_0M_0$	RE	4	A	5
26	M	74	$T_1N_0M_0$	ND	6	A	10

*For 26 patients with early tongue squamous cell carcinoma treated at Okayama University Hospital, Japan. †At time of initial surgery. ‡Site of primary tongue carcinoma. **Interval between surgery for primary tongue carcinoma and presentation of neck metastases. TNM = tumour–node–metastasis classification (according to the Union for International Cancer Control criteria) at presentation; F = female; LE = left edge of tongue; A = alive; RE = right edge of tongue; M = male; D = died of tongue carcinoma; ND = no data

relationship between HMGB1 expression and late neck metastasis in early tongue SCC.

HMGB1 and RAGE have been identified as a ligand–receptor pair that play an important role in regulating the invasiveness of tumour cells.[10] In addition, HMGB1 is considered to be a general regulator of cell migration.[9] HMGB1 is known to be a metastasis-associated gene. The administration of anti-HMGB1 antibodies was shown to suppress metastasis formation by Lewis lung tumour cells implanted subcutaneously in recipient mice.[9] Recently, HMGB1 overexpression has also been reported in several kinds of cancers in the head and neck region. Expression levels of HMGB1 were higher in a metastatic oral SCC cell line than those in a non-metastatic oral SCC cell line.[15] HMGB1 expression significantly affects the prognosis of patients with oesophageal SCC by regulating the expression of vascular endothelial growth factor

TABLE II
CRUDE ASSOCIATIONS BETWEEN DEMOGRAPHIC OR CLINICAL FACTORS AND LATE NECK METASTASIS*

Characteristic	*n*	Cases† (*n*)	Controls‡ (*n*)	Crude OR	95% CI
Sex					
– Male	15	8	7	5.14	0.82–32.30
– Female	11	2	9	1.00	
Age (years)					
– ≤63	11	5	6	1.67	0.34–8.26
– >63	15	5	10	1.00	
Tumour (T) classification**					
– T_1	16	5	11	1.00	
– T_2	10	5	5	2.00	0.43–11.22
Pathology					
– Well differentiated	15	5	10	1.00	
– Moderately to poorly differentiated	11	5	6	1.67	0.34–8.26
HMGB1 score					
–0–4	16	4	12	1.00	
–5–7	10	6	4	4.50	0.82–24.6

*For 26 patients with early tongue squamous cell carcinoma treated at Okayama University Hospital, Japan. †Late neck metastasis cases; $n = 10$. ‡Patients with no metastasis; $n = 16$. **Determined according to Union for International Cancer Control criteria. OR = odds ratio; CI = confidence interval

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TABLE III
POTENTIAL PROGNOSTIC FACTORS FOR LATE NECK METASTASIS*

Prognostic factor	Adjusted OR	95% CI	*p*
HMGB1 score (5–7 *vs* 0–4)	3.82	0.55–26.50	0.18
Sex (male *vs* female)	9.28	0.82–105.13	0.072
Tumour (T) classification (T_2 *vs* T_1)	5.97	0.53–64.92	0.15

*For 26 patients with early tongue squamous cell carcinoma treated at Okayama University Hospital, Japan. OR = odds ratio; CI = confidence interval

C to promote lymphangiogenesis and lymph node metastasis.[14] HMGB1 overexpression has been associated with tumour classification, node classification, distant metastasis and clinical stage in nasopharyngeal carcinoma.[12] Liu *et al*. concluded that HMBG1 protein may contribute to the malignant progression of head and neck SCC, and may provide a novel prognostic marker and potential therapeutic target for patients with head and neck SCC.[13]

Although male sex is sometimes mentioned as a factor associated with a high risk of occult metastasis in oral cancers,[16] sex generally does not influence prognosis in patients with tongue cancer.[17] The association seen in the present study may reflect not only sex itself, but sex differences in smoking prevalence.[18] In other words, sex partly explains the residual confounding as we did not directly adjust for smoking status.

- **Overexpression of HMGB1 has been reported in a variety of human cancers**
- **Few studies have investigated the relationship between HMGB1 expression and head and neck cancer prognosis**
- **This study examined whether late neck metastasis in early tongue squamous cell carcinoma (SCC) can be predicted by evaluating primary lesion HMGB1 expression**
- **HMGB1 expression may not be a strong predictor for late metastasis of early tongue SCC**

Risks of occult metastases and local recurrence have been reported as higher in patients with pathologically staged T_2 tumours than in those with pathologically staged T_1 tumours.[4] However, in the present study, status in terms of T_1 or T_2 did not appear to be an important factor for predicting late neck metastasis in crude analysis (odds ratio = 2.00). One possible explanation for this discrepancy may be our strict choice of patients (we only included those who underwent partial glossectomy as an initial treatment). Alternatively, as already discussed, HMGB1 may mask true strong relationships between tumour classification and neck metastasis. The association would then become clearer after adjusting for HMGB1.

As overexpression of HMGB1 is not always seen in all types of cancer, individual studies of HMGB1 levels in specific cancer types are needed.[19] More work is necessary to clarify whether HMGB1 is involved in the development and metastasis of tongue SCC.

Conclusion

No significant difference in HMGB1 expression level was identified between early tongue SCC patients with late neck metastasis and those without. More work will be necessary to clarify the relationship between HMGB1 expression and late neck metastasis in early tongue SCC.

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Address for correspondence:
Dr Yorihisa Orita,
Department of Otolaryngology,
Head and Neck Surgery,
Okayama University Graduate School of Medicine,
Dentistry and Pharmaceutical Sciences,
2-5-1 Shikata-Cho, Kita-Ku,
700-8558 Okayama, Japan

Fax: (+81)-86-235-7308
E-mail: y.orita@live.jp

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