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Elevated expression of HMGB1 in squamous-cell carcinoma of the head and neck and its clinical significance

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ABSTRACT

Purpose: HMGB1 overexpression has been reported in a variety of human cancers. However, the role of HMGB1 in squamous-cell carcinoma of the head and neck (SCCHN) remains unclear. The aim of the present investigation was to analyse HMGB1 protein expression in both SCCHN tissue and cell levels and to assess its prognostic significance in SCCHN.
Methods: HMGB1 protein expression in 103 primary SCCHN tissue specimens was analysed by immunohistochemistry and correlated with clinicopathological parameters and patient outcome. Additionally, HMGB1 protein expression was evaluated in cell level by Western blotting.
Results: By Western blotting analysis, all the 5 SCCHN cell lines overexpressed HMGB1 protein, whereas the non-transformed immortalised cell line NP-69 had relatively weak HMGB1 protein expression. Immunohistochemical staining revealed that HMGB1 protein was detected in 91 (91/103, 88.3%) primary tumour samples, but only in 7 (7/16, 43.75%) adjacent non-carcinoma samples ($p < 0.001$); moreover, HMGB1 overexpression was significantly associated with T classification ($p = 0.001$), clinical stage ($p < 0.001$), recurrence ($p < 0.001$) and lymph node metastasis ($p < 0.001$). Survival analysis demonstrated that high HMGB1 expression was significantly associated with shorter disease-free and overall survival (both $p < 0.001$), especially in late patients with SCCHN. When HMGB1 expression and lymph node status were combined, patients with HMGB1 overexpression/lymph node (+) had both poorer disease-free and overall survival than others (both $p < 0.001$). Multivariate analysis further demonstrated that HMGB1 was an independent prognostic factor for patients with SCCHN.
Conclusions: HMGB1 protein may contribute to the malignant progression of SCCHN, and present as a novel prognostic marker and a potential therapeutic target for patients with SCCHN.

1. Introduction

Squamous-cell carcinoma of the head and neck (SCCHN) is the sixth most frequently occurring malignancy worldwide, which represents a major international health problem. Annually, there are more than 500,000 new cases and 200,000 deaths around the world.[1] Despite improved detection and aggressive treatment approaches, including surgical

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resection and chemoradiation protocols, SCCHN is still a great threat to human life and limited improvement in 5-year survival has been achieved during the last few decades. The poor outcome has mainly been attributed to local and distant lymph node metastasis. The 5-year survival rate is less than 50% for patients with an ipsilateral lymph node metastasis and around 20% for patients with distant metastasis.[2] Therefore, the discovery of SCCHN-related metastasis genes and their mechanisms is of great importance for the development of novel strategies in the prevention and treatment of patients with SCCHN.

In our most recent investigation, some differentially expressed genes have been identified to be candidate biomarkers for laryngeal carcinoma using cDNA microarray approach, among which, HMGB1 was chosen for a detailed analysis.[3] HMGB1 is a non-histone chromosomal protein, which has been associated with a variety of biologically important processes including transcription, DNA repair, V(D)J recombination, differentiation, development and extracellular signalling.[4–6] As a nuclear protein, HMGB1 has the ability to induce bends in DNA and to promote the formation of nucleoprotein complexes, thus facilitating the interaction of DNA binding proteins with their cognate sites in chromatin.[7] In addition to its nuclear role, HMGB1 is also passively released from necrotic cells and is actively secreted by inflammatory cells, then functioning as an extracellular signalling molecule.[8]

Increased expression of HMGB1 has been reported in a panel of tumour types, such as gastrointestinal stromal tumours,[9] colorectal cancer,[10] prostate carcinoma[11,12] and nasopharyngeal carcinoma.[13] Overexpression of HMGB1 participates in a variety of processes involved in cancer progression, including apoptosis,[14] angiogenesis,[15] inflammatory microenviroment[16] and invasion,[17] indicating the significance of HMGB1 as a potential therapy target in human malignancies. However, there were little published reports evaluating the role of HMGB1 protein expression in SCCHN, particularly with respect to clinical outcome. Therefore, in order to gain better insight into the clinical relevance of HMGB1 protein in SCCHN, the present study was carried out to investigate HMGB1 protein expression in a larger number of archival SCCHN tissue samples and cell lines, and further to assess whether HMGB1 expression was correlated with clinicopathological parameters and prognosis in patients SCCHN.

2. Materials and methods

2.1. Patients and tissue preparation

A total of 103 patients with SCCHN, who underwent partial or total laryngectomy at the Department of Otolaryngology of Xiangya Hospital in Central South University from January 2002 to October 2004, were enrolled in this retrospective study. All patients had no history of previous malignancies, no history of radiotherapy or chemotherapy. Recurrence and metastasis were diagnosed by physical examinations, imaging evaluation, operation and postoperative pathological examinations. Informed consent was obtained from all patients before surgery, and this investigation was approved by the Research Ethics Committee of Central South University, Changsha, China.

Table 1 – Clinicopathological features of the studied 103 cases of SCCHN.

Variables	Number of patients	Percentage (%)
Age		
<58	47	45.6
⩾58	56	54.4
Sex		
Female	4	3.9
Male	99	96.1
Alcohol intake		
Yes	55	53.4
No	48	46.6
Smoking		
Yes	54	52.4
No	49	47.6
Tumour site		
Superaglottic	29	28.2
Glottic	63	61.2
Subglottic	1	1
Hypopharyngeal	10	9.7
Tumour grade		
G1	66	64.1
G2	21	20.4
G3	16	15.5
Tumour size		
T1	16	15.5
T2	36	35
T3	42	40.8
T4	9	8.7
Clinic stage		
I	15	14.6
II	26	25.2
III	40	38.8
IV	22	21.4
Lymph node metastasis		
N0	64	62.1
N+	39	37.9
Recurrence		
Yes	59	57.3
No	40	38.8

The main clinical and pathological variables of all patients were described in detail in Table 1. There were 99 male and 4 female patients, with a mean age of 57.86 years (range: 27–80 years, standard deviation (SD) = 10.348). According to the 2002 TNM classification of malignant tumours by the International Union Against Cancer,[18] 29 cases were supraglottic, 63 were glottic, 1 was subglottic and 10 were hypopharyngeal carcinomas. There were 15 cases in stage I (T1N0M0 15 cases), 26 cases in stage II (T2N0M0 26 cases), 39 cases in stage III (T3N0M0 19 cases, T1N1M0 1 cases, T2N1M0 5 cases, T3N1M0 14 cases) and 23 cases in stage IV (T2N2M0 5 cases, T3N2M0 7 cases, T3N3M0 1 case, T3N1M1 1 case, T4N0M0 4 cases, T4N1M0 3 cases and T4N2M0 2 cases). Considering pathological grading, 66 were staged as well-differentiated (G1), 21 as moderately differentiated (G2) and 16 as poorly differentiated (G3). Thirty-nine patients with lymph node metastasis were validated by conventional postoperative pathological examinations and 1 case had distant lung

metastasis according to imaging evaluation. Fifty-nine patients experienced tumour recurrence after surgery.

2.2. Immunohistochemistry

Immunohistochemical staining was performed using the PV-6001 Two-Step IHC Detection Reagent following the manufacturer's recommended protocol (ZhongShan Goldenbridge Bio, Beijing, China). Briefly, antigen retrieval was carried out in 10 mmol/l citrate buffer (pH 6.0) for 15 min at 100 °C in a microwave oven. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min at room temperature. Slides were incubated with HMGB1 rabbit polyclonal antibody (ab-18256, dilution 1:500) (Abcam, Cambridge, United States of America (USA)) at 4 °C overnight, followed by addition of HRP-labelled goat anti-rabbit polymers. Immunoreactive proteins were visualised with 3′,3′-diaminobenzidine and counterstained with Mayer's haematoxylin. Negative control slides were probed with normal goat serum under the same experimental conditions.

2.3. Evaluation of staining

Sections were evaluated and scored by two board-certified pathologists (Li Bo and Feng Xueping), who were blinded to the clinical parameters. For HMGB1 assessment, the entire tissue section was scanned to assign the scores. The staining intensity was scored as 0 (negative), 1 (weak), 2 (medium) and 3 (strong). The extent of staining was scored as 0 (0%), 1 (1–25%), 2 (26–50%), 3 (51–75%) and 4 (76–100%), according to the percentages of the positive staining areas in relation to the entire carcinoma-involved area or the entire section for adjacent non-carcinoma samples. The sum of the intensity and extent scores was used as the final staining score (ranges: 0–7) for HMGB1. Tumours were categorised into three groups (scored 0–3, 4–5 and 6–7) based on the final staining score and tumours with a final staining score of >3 were considered to be positive.[13] To analyse the prognosis between groups, the expression of HMGB1 was thus divided into low expression (scored 0–5) and high expression (scored 6–7).

2.4. Cell culture

The SCCHN cell line Tu686 was established from a primary tumour in the base of tongue. Derived through repeated *in vivo* selection in nude mice from a lymph node metastasis from the same patient, M2 and M4 were highly metastatic cell lines capable of generating high incidences of lymph node and lung metastasis.[19] Tu212 was established from a primary hypopharynx tumour. The above 5 SCCHN cell lines were kindly provided by Dr. Zhuo (Georgia) Chen (Emory University Winship Cancer Institute, Atlanta, Georgia). Additional SCCHN cell line Hep-2 was derived from larynx (trans glottis) and purchased from Central Experiment Laboratory of Xiangya Medical School, Central South University, Changsha, China. All cell lines were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (1:1) supplemented with 10% foetal bovine serum (FBS), 100 IU/ml penicillin and 100 IU/ml streptomycin at 37 °C in a humidified atmosphere with 5% CO_2.

2.5. Western blotting

The protein of non-transformed immortalised epithelium cell line (NP-69) and clinical sample of human colorectal carcinoma tissue was kindly provided by Cancer Research Institute, Xiangya School of Medicine, Central South University, Changsha. NP-69 protein (internal control), together with other 5 SCCHN cell line protein lysates, was quantified by bicinchoninic acid protein assay kit (Beyotime, China). Total protein (50 μg) was separated by 10% SDS–PAGE (v/v) and then transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The blotted membranes were incubated with rabbit poly-antibody against HMGB1 (ab-18256, dilution 1:1500) (Abcam, Cambridge, USA) at 4 °C overnight. After washing, they were incubated with HRP-labelled Goat Anti-rabbit IgG for 1 h at room temperature. Bands were finally visualised by employing the BeyoECL Plus Detection System (Beyotime, China). HMGB1 protein expression levels were quantified by FluorChem FC2 (San Leandro, CA) and represented as the densitometric ratio of the targeted protein to the housekeeping protein of β-actin (Beyotime, China). The experiments were repeated three times.

2.6. Follow-up

Follow-up materials after surgery were obtained from 99 (96.1%) patients, and 4 patients lost to follow-up because of telephone number changes or home moving. Recurrence and metastasis were diagnosed by clinical examination, imaging evaluation and pathological studies. Overall survival (OS) and disease-free survival (DFS) were calculated from the day of surgery to the date of death or that of tumour relapse. Deaths from other causes were treated as censored cases. The follow-up time ranged from 2 to 60 months, with a median follow-up time of 45.97 months (SD = 19.435).

2.7. Statistical analysis

Continuous variables were expressed as mean ± SD. Statistical significance between the expression of HMGB1 protein and clinicopathological parameters was compared by the χ^2 test. Survival analyses were undertaken using the Kaplan–Meier method and curves were compared by the log-rank test. The Cox's proportional hazards model was applied in the multivariate analysis to identify which factors were independent indicators for prognosis. All statistical analyses were performed with SPSS 17.0 software. A p-value < 0.05 was considered to be statistically significant.

3. Results

3.1. Increased HMGB1 protein expression in SCCHN tissue and cell levels

To investigate the protein expression profile of HMGB1 in SCCHN, immunohistochemistry was initially performed in 103 paraffin-embedded, archival SCCHN primary tumour samples and in 16 available adjacent non-carcinoma samples. Positive HMGB1 immunostaining was predominantly observed in the nuclei of carcinoma and non-carcinoma epithe-

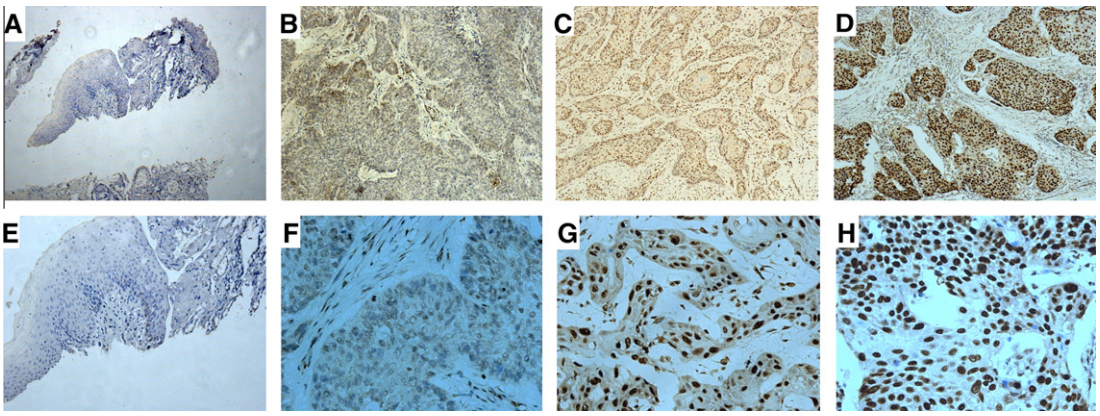

Fig. 1 – Representative immunohistochemical staining for HMGB1 in primary human SCCHN tissues and adjacent non-carcinoma epithelial tissues. Negative expression of HMGB1 in adjacent non-carcinoma epithelial tissues (A, E). HMGB1 protein staining in primary SCCHN specimens with different extent of final score: scored 7 (B, F); scored 5 (C, G); scored 2 (D, H) (original magnification A–D, ×100; E–H, ×400).

lial cells (Fig. 1). HMGB1 protein was detected in 91 (88.3%) primary tumour samples, but only in 7 (43.75%) normal samples ($p < 0.001$). Among tumour samples, HMGB1 expression was scored 0–3 in 12 (11.6%; Fig. 1B and F), 4–5 in 46 (44.7%; Fig. 1C and G) and 6–7 in 45 specimens (43.7%; Fig. 1D and H). However, HMGB1 expression was weaker in the adjacent non-carcinoma epithelial cells, although positive staining also existed (Fig. 1A and E).

Furthermore, HMGB1 protein expression in the cultured human SCCHN cell lines was also investigated by Western blotting. As presented in Fig. 2A and B, HMGB1 protein was detected in all the selected 5 SCCHN cell lines. More importantly, cell lines M2 and M4 with the highest metastatic ability revealed the strongest HMGB1 protein expression, and the other three poorly metastatic parental cell lines (Hep-2, Tu686, Tu212) demonstrated a relatively low HMGB1 protein expression, whereas the non-transformed cell line NP-69 used as a control demonstrated almost negative HMGB1 protein expression. HMGB1 expression levels in M4, M2, Hep-2, Tu212, Tu686 and NP-69 were 0.96, 0.92, 0.66, 0.87, 0.81 and 0.15 units (HMGB1/β-actin protein), respectively (Fig. 2B).

3.2. *Correlation between increased HMGB1 protein expression and clinicopathological parameters*

The association between HMGB1 protein expression and clinicopathological characteristics of SCCHN was explored by the χ^2 test. As summarised in Table 2, HMGB1 overexpression was significantly associated with tumour T classification ($p = 0.001$), advanced stage ($p < 0.001$), lymph node metastasis ($p < 0.001$) and recurrence ($p < 0.001$), respectively. However, no significant relationship existed between HMGB1 protein level and variables such as age ($p = 0.272$), alcohol history ($p = 0.624$), smoking ($p = 0.052$), tumour grade ($p = 0.154$) and tumour site ($p = 0.075$).

3.3. *Correlation between HMGB1 protein expression and patients' survival*

Ninety-nine patients with intact follow-up information (5 patients lost) were included in the survival analyses. As determined by the Kaplan–Meier method, the expression of HMGB1 in SCCHN was significantly correlated with disease-

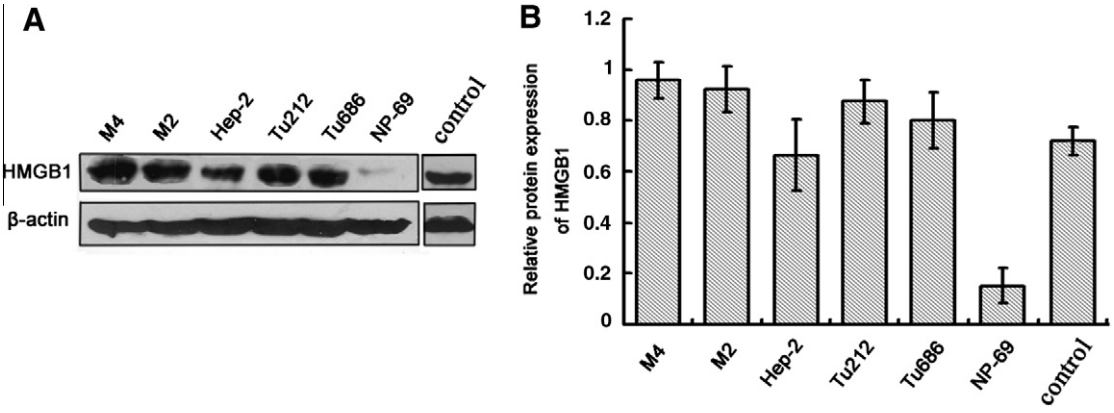

Fig. 2 – Representative Western blotting results of HMGB1 protein expression in the 5 selected human SCCHN cell lines and non-transformed immortalised cell line NP-69 and one clinical sample of human colorectal carcinoma tissue with definite HMGB1 protein expression (used as a positive control) (A). The abundance of HMGB1 protein was demonstrated relative to the levels of β-actin protein (B).

Table 2 – Correlations between HMGB1 protein expression and clinicopathological variables in patients with SCCHN.

Variables	Total	HMGB1 expression			*p*-Value*
		0–3	4–5	6–7	
Age					
<58	47	5	25	17	0.272
⩾58	56	7	21	28	
Alcohol intake					
Yes	55	6	27	22	0.624
No	48	6	19	23	
Smoking					
Yes	54	3	29	22	0.052
No	49	9	17	23	
Tumour grade					
G1	66	6	34	26	0.154
G2 + G3	37	6	12	19	
Tumour site					
Glottic	63	9	32	22	0.075
Others	40	3	14	23	
T classification					
T1 + T2	52	12	22	18	**0.001**
T3 + T4	51	0	24	27	
Clinical stage					
I–II	41	12	20	9	**<0.001**
III–IV	62	0	26	36	
Lymph node status					
N0	64	12	38	14	**<0.001**
N+	38	0	8	31	
Recurrence[a]					
Yes	59	1	24	34	**<0.001**
No	40	11	19	10	

* *Note:* $p \leqslant 0.05$ was considered to be statistically significant.

[a] Four patients lost to follow-up because of telephone number changes or home moving.

free survival ($p < 0.001$; Fig. 3A) and overall survival ($p < 0.001$; Fig. 3B). The log-rank test further demonstrated that the survival time was significantly different between groups with high and low expression of HMGB1 protein, indicating that high level of HMGB1 was tightly correlated with a shorter survival time. Multivariate analysis was also performed with the Cox proportional hazards model including age, grade, tumour site, T classification, clinical stage, metastasis and HMGB1 expression. The results showed that HMGB1 protein expression had a significant correlation with SCCHN prognosis and was found to be an independent prognostic factor of outcomes in patients with SCCHN after tumour resection (hazard ratio = 2.133, 95% confidence interval (CI) = 1.079–4.218, $p = 0.029$) (Table 3).

The prognostic value of HMGB1 protein expression in selective patient subgroups, stratified according to the tumour clinical stage was also analysed. In the early stage group (I + II), no statistically significant difference was found in disease-free survival ($p = 0.242$; Fig. 4A) and overall survival ($p = 0.196$; Fig. 4B). While in the late stage group (III + IV), patients with a high level of HMGB1 protein expression had an obviously shorter disease-free survival and overall survival time when compared with patients with a low level of HMGB1 protein expression ($p = 0.005$; $p < 0.001$; Fig. 4C and D). Thus, HMGB1 protein expression pattern might be a valuable prognostic marker for late stage patients with SCCHN.

It has been reported that lymph node metastasis was an important prognostic factor in patients with SCCHN.[20] Thus, we investigated the correlation between lymph node metastasis and patients prognosis in SCCHN. The results revealed that lymph node metastasis was significantly associated with short disease-free and overall survival ($p < 0.001$; $p < 0.001$, respectively; Fig. 5A and B) and was an independent prognostic factor for overall survival by multivariate analysis ($p = 0.045$; Table 3). Therefore, a subset analysis was carried out by combining HMGB1 expression with lymph node status. The results demonstrated that patients with the phenotype of high HMGB1 expression/lymph node (+) had poorer disease-free and overall survival than that of others ($p < 0.001$; $p < 0.001$, respectively; Fig. 5C and D).

4. Discussion

In the current study, we investigated the protein expression of HMGB1 both in SCCHN cell lines and in a series of 103 clinical

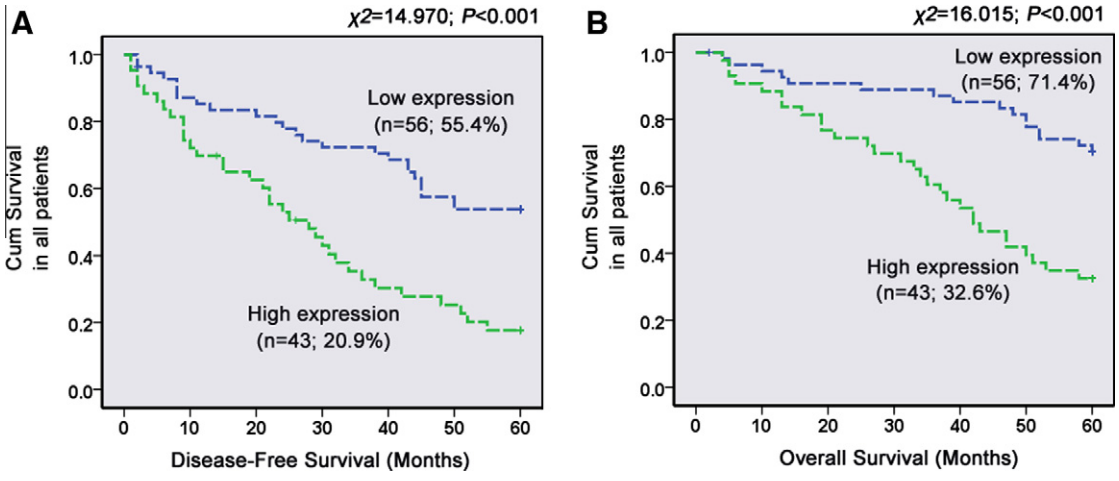

Fig. 3 – Kaplan–Meier survival analysis of disease-free survival (A) and overall survival (B) in all patients according to HMGB1 protein expression. The log-rank test was applied to calculate *p*-value.

Table 3 – Multivariate Cox model analysis of disease-free and overall survival.

Characteristics	Disease-free survival		Overall survival	
	Hazard ratio (95% CI)	*p*-Value	Hazard ratio (95% CI)	*p*-Value
Age (<58/⩾58)	0.960 (0.545–1.689)	0.886	0.825 (0.415–1.637)	0.582
Grade (G1/G2, G3)	1.033 (0.596–1.790)	0.908	1.393 (0.746–2.600)	0.298
T classification (T1 + T2/T3 + T4)	0.698 (0.313–1.559)	0.381	1.268 (0.544–2.953)	0.582
Site (glottic/others)	0.604 (0.317–1.152)	0.126	0.613 (0.280–1.341)	0.221
Clinical stage (I–II/III–IV)	2.846 (0.923–8.772)	0.069	2.252 (0.547–9.278)	0.261
Metastasis (±)	1.138 (0.512–2.533)	0.751	2.771 (1.025–7.491)	**0.045**
HMGB1 expression (high/low)	2.121 (1.191–3.776)	**0.011**	2.133 (1.079–4.218)	**0.029**

Note: All the clinicopathological variables listed in table were included in the multivariate analysis.
p-Values in bold were statistically significant.
Abbreviation: 95% CI, 95% confidence interval.

paraffin-embedded specimens with intact follow-up information. Immunostaining results revealed that HMGB1 protein was obviously higher in SCCHN tissues compared with adjacent non-carcinoma tissues. We also demonstrated that HMGB1 overexpression related to disease progression and survival, and that HMGB1 might provide independent information to guide prediction of the clinical outcome in patients with SCCHN.

The present study breaks new ground in linking HMGB1 expression with SCCHN. Our results were in agreement with previous studies of other malignancies, such as gastrointestinal stromal tumours,[9] colorectal cancer,[10] prostate carci-

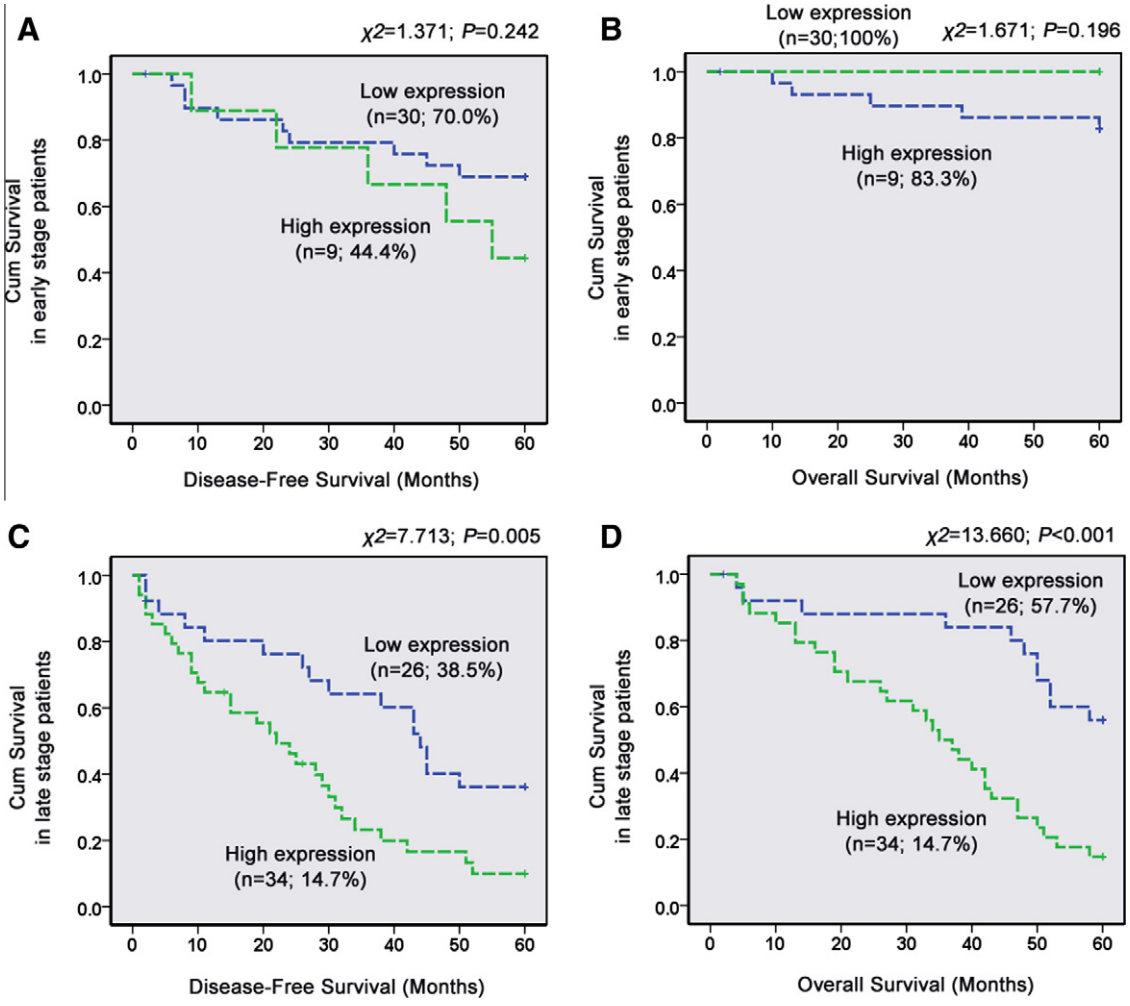

Fig. 4 – Kaplan–Meier survival analysis of disease-free survival patients in early stage (I + II) (A) and in late stage (III + IV) patients (C) according to HMGB1 protein expression; analysis of overall survival in early stage (B) and in late stage patients (D) according to HMGB1 protein expression. The log-rank test was used to calculate *p*-value.

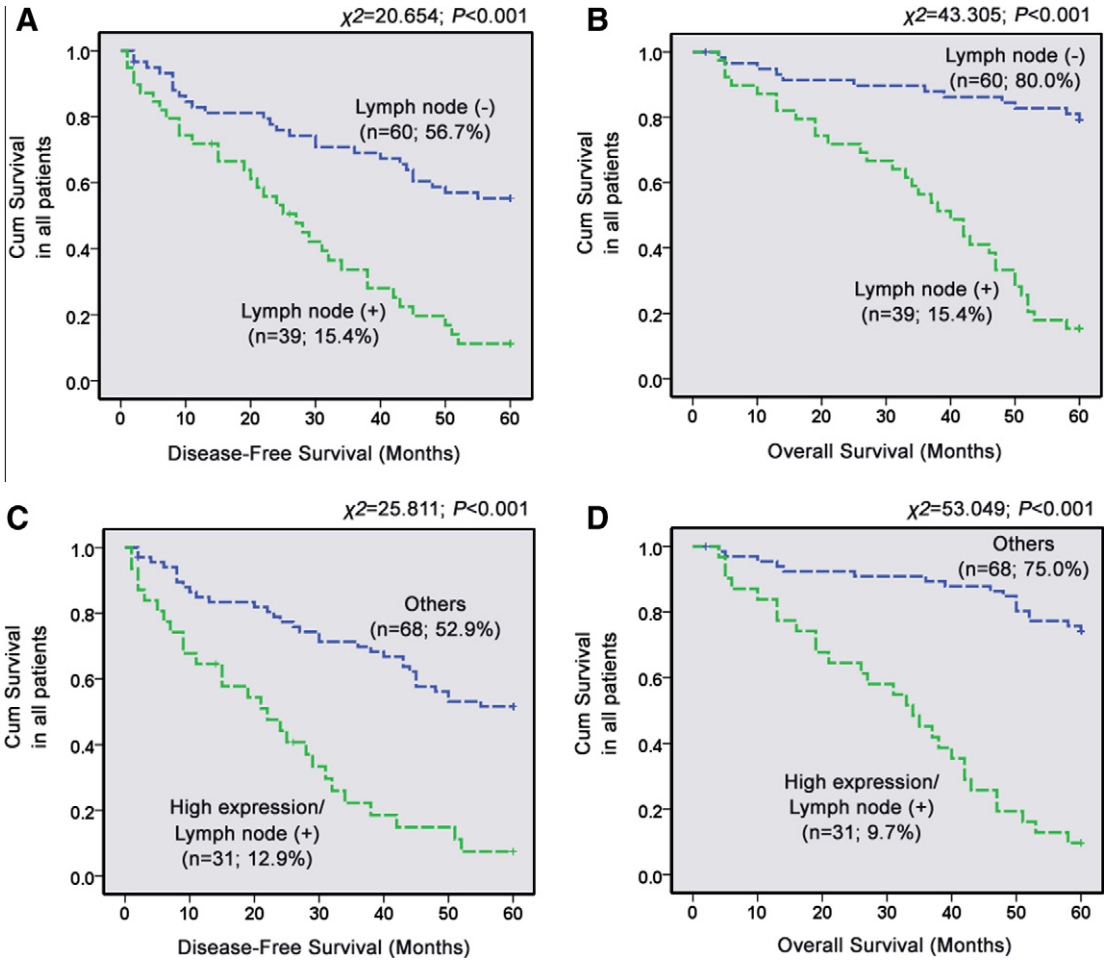

Fig. 5 – Kaplan–Meier survival analysis of disease-free survival (A) and overall survival (B) in all patients according to the lymph node status; survival analysis of disease-free survival (A) and overall survival (B) in all patients according to HMGB1 expression/lymph node status. The log-rank test was applied to calculate *p*-value.

noma[11,12] and nasopharyngeal carcinoma,[13] in which overexpression of HMGB1 in tumour cells has been observed and a correlation between HMGB1 expression and tumour malignant phenotype, such as metastasis and recurrence, has been established. In this respect, HMGB1 can be considered as an oncoprotein and overexpression of HMGB1 is associated with each of central hallmarks of cancer including migration, proliferation, angiogenesis, apoptosis, invasion and metastasis.[21]

Different models exist which would explain the beneficial influence of HMGB1 on tumour development. HMGB1 proteins are constitutively expressed in the nucleus of tumour cells, and also can be released by inflammatory cells and by tumour cells undergoing necrosis or triggered by hypoxia, nutrient deprivation, absence of essential growth factors or application of conventional anticancer therapy.[16,22] Intranuclearly, HMGB1 binds without sequence specificity to the minor groove of DNA, which stabilises nucleosome formation, regulates gene transcription (p53, p73, retinoblastoma protein, etc.)[23–25] and the activity of steroid hormone receptors.[26] On the other hand, extracellular HMGB1 induces cancer cell growth, mobility, invasion and metastasis via binding to specific membrane receptors including the receptor for advanced glycation end products (RAGE), and then activating key cell signalling pathways, such as MAPK and NF-κB[27,28]; blockade of the RAGE–HMGB1 interaction suppresses tumour growth and metastasis.[29] Moreover, constant release of HMGB1 as a proinflammatory cytokine from necrotic tumour cells would create a microenvironment similar to chronic inflammations, and this condition was known to contribute to the development of epithelial malignancies.[30]

With regard to the prognostic value of HMGB1 protein in patients with SCCHN, it remains unclear until now. Currently, prognostic evaluation is mainly based on traditional methods including the clinical stage, tumour site and histopathological grade. Recent studies have suggested that other factors, such as molecular and cellular characteristics of the primary tumours, may improve our ability to prognosticate.[31] In our present investigation, HMGB1 protein expression was inversely correlated with overall survival and disease-free survival. In particular, HMGB1 expression was significantly associated with a poor prognosis in late stage patients with SCCHN. Previous studies indicated that lymph node metastasis could be used as a prognostic factor for patients with SCCHN,[20] which was consistent with our present study. Therefore, a subset-combined survival analysis was carried out by both HMGB1 protein expression and the lymph node status. The results re-

vealed that patients with the phenotype of high HMGB1 expression/lymph node metastasis (+) had both shorter disease-free and overall survival time than patients with other phenotypes. Therefore, the evaluation of HMGB1 protein alone and/or together with the lymph node status may further provide new information for patients' prognosis. Also, such information could provide better planning of appropriate treatment strategies, especially for the determination of the neck dissection as well as for the better management after a surgery.

In conclusion, our current study indicated that HMGB1 was up-regulated in human SCCHN and that HMGB1 overexpression was significantly correlated with tumour malignant progression and poor survival in patients with SCCHN, which suggested that HMGB1 might serve as a specific and a novel prognostic marker in SCCHN. Furthermore, given the broad expression of HMGB1 in SCCHN especially in late stage patients with SCCHN, and the antitumour effects of HMGB1 modulation *in vitro* described by others,[32,33] HMGB1 presents as an attractive therapeutic target. However, further studies will be required to determine the molecular mechanism of HMGB1 involved in SCCHN progression and prognosis, which may lead to further development of new approaches targeting HMGB1 for effective tumour management.

Conflict of interest statement

None declared.

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