Loss of epithelial membrane protein-2 expression confers an independent prognosticator in nasopharyngeal carcinoma: a cohort study

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Complete List of Authors:	Chen, Yi-Hsien; Department of Emergency Medicine, Chi-Mei Medical Center Wu, Li-Ching; Department of Pathology, Chi-Mei Medical Center Wu, Wen-Ren; Institute of Biomedical Science, National Sun Yat-sen University Lin, Hung-Jung; Department of Emergency Medicine, Chi-Mei Medical Center Lee, Sung-Wei; Department of Radiation Oncology, Chi-Mei Medical Center Lin, Ching-Yih; Deparment of Otolaryngology, Chi-Mei Medical Center Chow, Nan-Haw; Department of Pathology, National Cheng Kung University Hospital Huang, Hsuan-Ying; Department of Pathology, Chang Gung Memorial Hospital-Kaohsiung Medical Center, Chang Gung University Li, Chien-Feng; Department of Pathology, Chi-Mei Medical Center Shiue, Yow-Ling; National Sun Yat-sen University, Institute of Biomedical Science
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Loss of epithelial membrane protein-2 expression confers an independent prognosticator in nasopharyngeal carcinoma: a cohort study

Yi-Hsien Chen, [1,#] Li-Ching Wu, [2,#] Wen-Ren Wu, [3] Hung-Jung Lin, [1] Sung-Wei Lee, [4] Ching-Yih Lin,[5] Shih-Lun Chang, [6] Nan-Haw Chow, [7,8] Hsuan-Ying Huang, [9] Chien-Feng Li, [2,3,10,11] Yow-Ling Shiue [3,*]

[1] Department of Emergency Medicine, Chi-Mei Medical Center, Tainan, Taiwan
[2] Department of Pathology, Chi-Mei Medical Center, Tainan, Taiwan
[3] Institute of Biomedical Science, National Sun Yat-sen University, Kaohsiung, Taiwan
[4] Department of Radiation Oncology, Chi-Mei Medical Center, Liouying, Tainan, Taiwan
[5] Division of Gastroenterology and Hepatology, Department of Internal Medicine, Chi-Mei Medical Center, Tainan, Taiwan
[6] Department of Otolaryngology, Chi-Mei Medical Center, Tainan, Taiwan
[7] Department of Pathology, National Cheng Kung University Hospital, Tainan, Taiwan
[8] Institute of Basic Medical Sciences, National Cheng Kung University, Tainan, Taiwan
[9] Department of Pathology, Chang Gung Memorial Hospital-Kaohsiung Medical Center, College of Medicine, Chang Gung University, Kaohsiung, Taiwan
[10] National Institute of Cancer Research, National Health Heath Research Institute, Tainan, Taiwan
[11] Department of Biotechnology, Southern Taiwan University, Tainan, Taiwan

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[#] These authors contributed equally

Correspondence to: Yow-Ling Shiue, Institute of Biomedical Science, National Sun Yat-sen University, Kaohsiung, Taiwan. E-mail: ylshiue@mail.nsysu.edu.tw

ABSTRACT

Background: Nasopharyngeal carcinoma (NPC) is an endemic head and neck epithelial malignancy in Southeastern Asia and Taiwan. Human epithelial membrane protein-2 gene (*EMP2*) is a highly conserved member of four-transmembrane (tetraspan) superfamily, which involves in the regulation of cell growth and differentiation.

Objective: To evaluate the expression of EMP2 protein and its clinicopathological associations in patients with NPC.

Design: Immunoexpression of EMP2 was retrospectively assessed biopsies of 124 consecutive NPC patients without initial distant metastasis and treated with consistent guidelines. The outcomes were correlated with clinicopathological features and patient survivals.

Results Loss of EMP2 expression (49.2%) was correlated with advanced primary tumor (p = 0.044), nodal status (p = 0.045) and the 7th American Joint Committee on Cancer (AJCC) stage (p = 0.027). In multivariate analyses, loss of EMP2 expression emerged as an independent prognosticator for worse disease-specific survival (DSS; p = 0.015) and local recurrence-free survival (LRFS; p = 0.030), along with AJCC stage III-IV (p = 0.034, DSS; p = 0.023, LRFS).

Conclusions Loss of EMP2 expression is common and associated with adverse prognosticators, and might confer tumor aggressiveness through hampering its interaction with specific membrane protein(s) and hence, the downstream signal transduction pathway(s).

Introduction

Nasopharyngeal carcinoma (NPC) is an endemic head and neck epithelial malignancy in Southeastern Asia and Taiwan; strongly linked to Epstein-Barr virus.[1 2] The latter association is especially authentic for the differentiated and undifferentiated non-keratinizing carcinoma types, according to current World Health Organization tumor classification, although genetic and environmental factors also play certain roles in pathogenesis.[1-3] The advances in diagnostic imaging, radiation therapy, and adjuvant chemotherapy of NPC have achieved better locoregional control, while it appears less satisfactory in final treatment outcomes.[4 5] Even though being an important parameter, TNM staging still has space to improve in terms of providing the optimal prognostication to the patients. [1 4-6] Therefore, to identify potential biomarkers with better correlation to tumor growth and/or treatment outcomes in patients with NPC, subsequently, to aid in risk stratification and perhaps development of therapeutic targets, are indispensable.

Human epithelial membrane protein-2 gene (*EMP2*), mapped to chromosome 16, is highly conserved across vertebrates.[7-9] The expression pattern of *EMP2* partially overlaps to that of the peripheral myelin protein 22 (*PMP22*, also known as the growth arrest-specific-3, *GAS3*) transcript. By containing the claudin domain and sharing approximately 40% amino acid identity with PMP22/GAS3,[10] the EMP2 protein was detected as a novel member of this four-transmembrane (tetraspan) superfamily.[11] In humans, EMP2 has a discrete cell type and tissue distribution, with high levels observed in the lung and moderate levels in the eye, heart, thyroid, uterus and intestine. [10 12 13] Functionally, the best understood tetraspan proteins are connexins, which form the major structural element of gap junctions. Connexins play important roles in the regulation of cell growth and differentiation. Cancer cells usually have downregulated levels of gap junctions, and several lines of evidence suggest that loss of gap junctional intercellular communication is an important step in carcinogenesis. Reexpression

of connexins in cancer cells causes normalization of cell growth control and reduced tumor growth.[14 15] Accordingly, we aimed to systematically analyze EMP2 immunoexpression in patients with NPC and identified that loss of EMP2 expression is associated with adverse prognosticators, conferring to poor survivals.

MATERIALS AND METHODS

Patients and tumor specimens

The institutional review board approved the study by using formalin-fixed tissue of NPC for this study (IRB100-09-003). Available paraffin-embedded tissue blocks were retrieved from 124 NPC patients who underwent biopsy between Jan 1993 and Dec 2002. These patients were free of distant metastasis at initial presentation. The histological subtypes were reappraised according to the current World Health Organization classification and, the tumor staging was reevaluated with the 7th American Joint Committee on Cancer (AJCC) system by two pathologists, independently.

Immunohistochemical staining and assessment of EMP2 expression

Tissue sections of 3-µm thickness were cut onto precoated slides from paraffin-embedded tissue blocks and were next routinely deparaffinized with xylene and rehydrated with ethanol washes. Slides were heated by the microwave in a 10 mM citrate buffer (pH 6.0) for 7 min to retrieve antigens. Endogenous peroxidase was blocked with 3% H_2O_2. Slides were next washed by Tris-buffered saline for 15 min and subsequently incubated with a rabbit polyclonal primary antibody targeting EMP2 (Atlas Antibodies, Stockholm, Sweden) at a dilution of 1:75 for 1 h. Primary antibodies were detected using the DAKO ChemMate EnVision Kit (K5001, Carpinteria, CA, USA). The slides were incubated and developed with the secondary antibody for 30 min, and 3,3-diaminobenzidine for 5 min, followed by counterstained using Gill's Hematoxylin. Immunoexpression of EMP2 was scored by two

pathologists (CF Li and HY Huang) using a multiheaded microscope to reach a consensus for each case without prior knowledge of clinical and follow-up information. The percentage of tumor cells with EMP2 immunoexpression was recorded for each specimen and loss of EMP2 expression (negative) was defined in cases with staining ≤ 5% tumor cells (see Statistical analysis).

Treatment and follow-up

All 124 patients with follow-up for outcome have received complete course of radiotherapy (RT, total dose ≥ 7,000 cGy) and also cisplatin-based chemotherapy in those of stage II-IV diseases, based on the previously published protocol.[16] The method of RT was in general uniform within this period. All patients were regularly monitored after RT until death or their last appointment with the mean follow-up duration being 59.6 months (range: 4-117).

Statistical analysis

Statistics were performed using SPSS 14.0 software (SPSS Inc., Chicago, IL, USA). Chi-square test was used to compare the EMP2 expression status and various clinicopathological parameters. The endpoints analyzed were disease-specific survival (DSS) and local recurrence-free survival (LRFS), calculated from the starting date of RT to the date of event developed. Patients lost to follow-up were censored on the latest follow-up date. Survival curves were plotted using the Kaplan-Meier method, and the log-rank test was performed to evaluate prognostic differences between groups. Multivariate analysis was carried out by the Cox proportional hazards model. However, as a component factor of the AJCC stage, primary tumor (T) and nodal status (N) was not introduced in multivariate comparisons. After testing a series of cutoff values in 5% increment, EMP2 expression was construed as negative when the expression index was ≤ 5% tumor cells. For all analyses, two-sided tests of significance were used with $p < 0.05$ considered significant.

RESULTS

Immunohistochemical expression of EMP2 and associations with clinicopathological variables in NPC specimens

As shown in Table 1, 124 cases of NPC consisted of five keratinizing squamous cell carcinomas, 54 non-keratinizing differentiated carcinomas, and 65 non-keratinizing, undifferentiated carcinomas. A total of 95 males and 29 females with a mean age of 48.6 years (range, 20-83) included. Seven cases were classified as stage I, 31 as Stage II, 46 as Stage III, and 40 as Stage IV. Immunoexpression of EMP2 was observed and successfully scored in all cases. Tumor-adjacent normal respiratory epithelium (Figure 1A) or non-tumor epithelium with squamous metaplasia (Figure 1B) could be appreciated in 71 samples and all showed intense EMP2 immunoexpression. A wide range of stained tumor cell, characterized by cytoplasmic and/or membranous staining, varying from 0-90% (median, 30%) were detected in tumor elements. Of these, 63 cases showed characteristic EMP2 staining (> 5% tumor cells; Figure 1C), while 61 cases were less than 5% staining and therefore classified as EMP2 negative (Figure 1D). Loss of EMP2 expression was significantly associated with cases featuring increment of primary tumor ($p = 0.004$), nodal status ($p = 0.045$) and AJCC stage ($p = 0.027$) (Table 2). However, no association between the EMP2 expression level and other clinicopathological factors was found.

Prognostic impact of EMP2 expression in NPC

Patients with NPC more frequently progressed to disease-specific mortality with N2-N3 nodal status ($p = 0.002$) and stage III-IV ($p = 0.007$) (Table 3). Besides, patients with advanced AJCC stage III-IV held shorter DSS ($p = 0.07$; Figure 2A) and LRFS ($p = 0.06$; Figure 2B). The development of local recurrence was significantly associated with T3-T4 ($p = 0.027$), N2-N3 status ($p = 0.023$) and AJCC stage III-IV ($p = 0.005$) with a medium duration of 24

months (Table 3). Of note, EMP2 negative correlated to a more aggressive clinical course with a significantly shorter DSS (p = 0.002; Figure 2C) and LRFS (p = 0.005; Figure 2D) in patients with NPC. In multivariate analysis (Table 4), loss of EMP2 expression steady remained as a robust prognosticator for both inferior DSS [p = 0.015, hazard ratio (HR) = 1.969] and worse LRFS (p = 0.030, HR = 2.136), following tumor stage (p = 0.034, HR = 2.115; p = 0.023, HR = 3.046, for DSS and LRFS, respectively).

DISCUSSION

Loss of EMP2 immunostaining as one potent prognosticator for both DSS and LRFS in a subset of patients with NPC was sustained in this study. However, significantly high EMP2 expression was found in ovarian cancer,[17] and was identified as an early predictor of endometrial cancers with unfavorable outcome.[18 19] Due to non-neoplastic peritoneal surface tissues were complete negative for EMP2 staining, thus EMP2 was regarded as increased expression in tumor cells in ovarian cancer.[17] Moderately intense, diffuse immunohistochemical stainings of tumor cell cytoplasm were identified in endometrioid adenocarcinoma, serous carcinoma, mixed endometrioid and serous carcinoma, mixed endometrioid and clear cell carcinoma.[18] On the other hand, compared to undifferentiated ones, predominant expressions of EMP2 in cytoplasm and/or membrane of squamous metaplasias and non-keratinizing NPCs were found in our study, suggesting that loss of EMP2 expression might change its interactions with some membrane proteins in NPC. Surface expression of the α6β1 integrin was specifically increased by EMP2 in NIH3T3 fibroblasts.[20] Moreover, surface expression and trafficking of integrin αvβ3 during the window of implantation, which are essential for endometrial-blastocyst interaction in mice, were affected by the EMP2 level and the interaction between EMP2 and focal adhesion kinase.[18 21 22] In mammals, 18 α and eight β subunits assemble into 24 different integrins,

which bind collagens, laminins, or arginine-glycine-aspartic acid-containing proteins. Integrins are regulated by conformational changes, clustering and trafficking, and regulatory mechanisms differ strongly between individual integrins and between cell types. Defective integrin activation or integrin signaling is associated with an array of pathological conditions.[23] Endocytosis and recycling are crucial in the regulation of integrin turnover and redistribution in adherent cells, especially during dynamic processes such as migration and invasion.[24] Therefore, EMP2 probably plays a tumor suppressor role through interacting with specific integrin(s) in epithelial cells and thereafter, manages regular signaling transduction in benign conditions.

In keep with the above finding, we uncovered that ectopic expression of *EMP2* in a malignant human urothelial cell line, J82, significantly reduced cell proliferation, cell cycle progression, migration and invasion in vitro (unpublished). Consistently, suppression subtractive hybridization isolated mouse ortholog *Emp2*, which sup-presses B-cell lymphoma tumorigenicity through a functional tumor suppressor phenotype.[9] Retroviral overexpression of *Emp2* in a malignant variant cell line derived from spontaneous in vitro outgrowth of splenic lymphocytes increased allogeneic cytotoxic T-lymphocyte susceptibility in *Emp2*-deficient mouse cells.[13] Constitutive overexpression of EMP2 or other epithelial membrane proteins including EMP1, EMP3 and PMP22, in human HEK293 epithelial cells led to purinergic receptor P2X, ligand-gated ion channel, 7 (P2RX7)-mediated cell blebbing, annexin V binding (phosphatidylserine exposure on the extracellular leaflet of the membrane), and cell death, through a caspase-dependent pathway. The C-terminal domain of P2RX7 protein associates with EMPs and mediates some aspects of the downstream signaling following P2RX7 activation.[25] All of these studies supported our clinical observations, reinforcing that EMP2 might play distinct characteristics in different cellular contexts. Indeed, the etiology of NPC is complex, including a host of viral, genetic and environmental

factors.[14 26 27] In spite of cure for the majority of the patients, challenges still exist in the prevention of disease relapse and treatment of patients with refractory or metastatic NPC.[28-30] Therefore, for the first time, loss of EMP2 expression was identified as a biomarker independently correlated with tumor aggression to facilitate appropriate allocation of adjuvant therapy, suggesting its significance for patient-tailored strategy to manage high-risk NPCs.

Except for loss of EMP2 expression, significantly increased hazard ratios of DSS and LRFS in NPC patients with higher stages (III-IV) were further ascertained, analogous to other studies.[31-33] Additionally, we revealed significant correlations between loss of EMP2 expression and primary tumor, nodal status and stage in NPCs, indicating its prospective role in preventing NPC progression and aggressiveness. Although the precise characteristics of the EMP2 protein in NPC progression remain to be elucidated, the potential utility of EMP2 immunostaining as a prognostic biomarker in NPCs is assured.

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Competing Interests The authors declare no competing financial or publication interests.

Contributors Huang HH, Li CF, Chow NW & Shiue YL contributed to experimental conception, design, immunohistochemical staining and analyses; Chen YH, Lin HJ, Lee SW, Lin CY & Chang SL acquired and analyzed the case history; Li CF & Shiue YL drafted the article and revised it critically for important intellectual content.

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Table 1. Clinical pathological features of 124 nasopharyngeal carcinomas

Variable	*n* (%)
Gender	
Male	95 (76.6)
Female	29 (23.4)
Age (years)	
< 60	98 (79.0)
≥ 60	26 (21.0)
Primary tumor (T)	
T1	30 (24.2)
T2	50 (40.3)
T3	21 (16.9)
T4	23 (18.5)
Nodal status (N)	
N0	24 (19.4)
N1	32 (25.8)
N2	48 (38.7)
N3	20 (16.1)
Stage	
I	7 (5.6)
II	31 (25.0)
III	46 (37.1)
IV	40 (32.2)
Histological grade	
Keratinizing	5 (4.0)
Non-keratinizing/differentiated	54 (43.5)
Non-keratinizing/undifferentiated	65 (52.4)
EMP2 expression level	
Positive (> 5% tumor cells)	63 (50.8)
Negative (≤ 5% tumor cells)	61 (49.2)

Table 2. Expression level of EMP2 and correlations with clinicopathologic variables (n = 124)

Variable	EMP2 expression score		p-value
	Positive (> 5% tumor cells)	Negative (≤ 5% tumor cells)	
Gender			0.926
Male	43	52	
Female	20	9	
Age (years)			0.926
< 60	50	48	
≥ 60	13	13	
Primary tumor (T)			0.044*
T1-T2	46	34	
T3-T4	17	27	
Nodal status (N)			0.045*
N0-N1	34	22	
N2-N3	29	39	
Stage			0.027*
I-II	25	13	
III-IV	38	48	
Histological grade			0.879
Keratinizing	3	2	
Non-keratinizing/differentiated	28	26	
Non-keratinizing/undifferentiated	32	33	

*, Statistically significant

Table 3. Univariate log-rank analysis of EMP2 expression score on survival outcome (*n* =124)

Variable	*n*	DSS [1]		LRFS [2]	
		n	*p*-value	*n*	*p*-value
Gender			0.878		0.346
Male	95	45		30	
Female	29	14		7	
Age (years)			0.996		0.755
< 60	98	48		29	
≥ 60	26	11		8	
Primary tumor (T)			0.065		0.027*
T1-T2	80	32		19	
T3-T4	44	27		18	
Nodal status (N)			0.002*		0.023*
N0-N1	56	18		12	
N2-N3	68	41		25	
Stage			0.007*		0.005*
I-II	38	10		3	
III-IV	86	49		32	
Histological grade			0.157		0.900
Keratinizing/Non-keratinizing	47	40		15	
Undifferentiated	77	39		22	
EMP2 expression level			0.002*		0.005*
Positive (> 5% tumor cells)	63	21		13	
Negative (≤ 5% tumor cells)	61	38		24	

*, Statistically significant; [1] DSS, disease-specific survival; [2] LRFS, local recurrence-free survival

Table 4. Multivariate survival analysis of EMP2 expression level on survival outcome

Variable	DSS [1]		LRFS [2]	
	HR[3] (95% CI[4])	*p*-value	HR[3] (95% CI[4])	*p*-value
AJCC Stage		0.034*		0.023*
I-II	1		1	
III-IV	2.115 (1.057-4.232)		3.046 (1.171-7.919)	
EMP2 expression level		0.015*		0.030*
Positive (> 5% tumor cells)	1		1	
Negative (≤ 5% tumor cells)	1.969 (1.144-3.391)		2.136 (1.076-4.237)	

*, Statistically significant; [1] DSS, disease-specific survival; [2] LRFS, local recurrence-free survival; [3] HR, hazard ratio; [4] CI, confidence interval

Figure legends

Figure 1 Immunohistochemically, non-tumor respiratory epithelium (A) and those with squamous metaplasia (B), demonstrate diffuse and strong EMP2 immunoexpression, which can also be appreciated in representative non-keratinizing carcinoma (C) but not in undifferentiated one (D).

Figure 2 Kaplan-Meier plotting illustrates the prognostic significance of tumor stage for (A) disease-specific survival (DSS) and (B) local recurrence-free survival (LRFS), respectively. The predictive value of EMP2 expression is also demonstrated (C, D).

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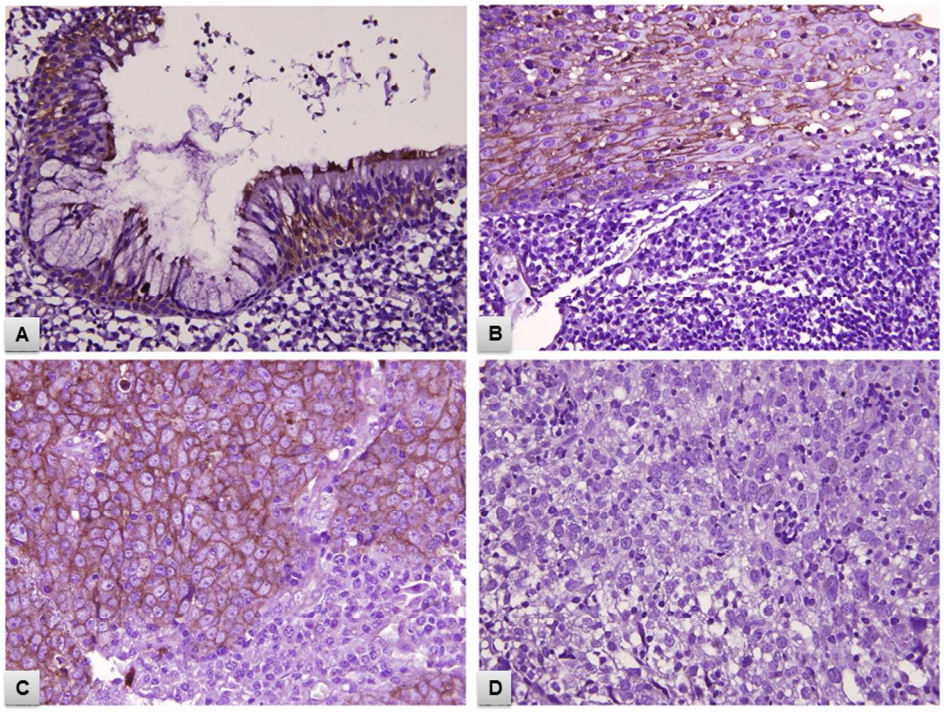

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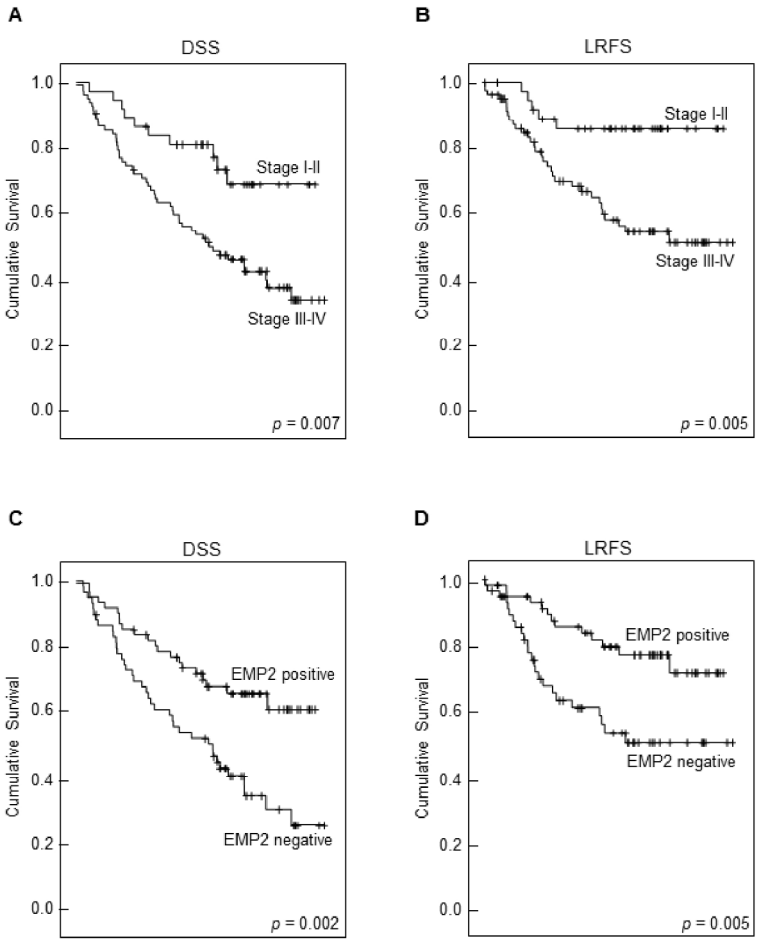

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STARD checklist for reporting of studies of diagnostic accuracy

(version January 2003)

Section and Topic	Item #		On page #
TITLE/ABSTRACT/ KEYWORDS	1	Identify the article as a study of diagnostic accuracy (recommend MeSH heading 'sensitivity and specificity').	1/2/1
INTRODUCTION	2	State the research questions or study aims, such as estimating diagnostic accuracy or comparing accuracy between tests or across participant groups.	3-4
METHODS			
Participants	3	The study population: The inclusion and exclusion criteria, setting and locations where data were collected.	4
	4	Participant recruitment: Was recruitment based on presenting symptoms, results from previous tests, or the fact that the participants had received the index tests or the reference standard?	4
	5	Participant sampling: Was the study population a consecutive series of participants defined by the selection criteria in item 3 and 4? If not, specify how participants were further selected.	4
	6	Data collection: Was data collection planned before the index test and reference standard were performed (prospective study) or after (retrospective study)?	2
Test methods	7	The reference standard and its rationale.	4
	8	Technical specifications of material and methods involved including how and when measurements were taken, and/or cite references for index tests and reference standard.	4-5
	9	Definition of and rationale for the units, cut-offs and/or categories of the results of the index tests and the reference standard.	5
	10	The number, training and expertise of the persons executing and reading the index tests and the reference standard.	5
	11	Whether or not the readers of the index tests and reference standard were blind (masked) to the results of the other test and describe any other clinical information available to the readers.	5
Statistical methods	12	Methods for calculating or comparing measures of diagnostic accuracy, and the statistical methods used to quantify uncertainty (e.g. 95% confidence intervals).	5
	13	Methods for calculating test reproducibility, if done.	5
RESULTS			
Participants	14	When study was performed, including beginning and end dates of recruitment.	4
	15	Clinical and demographic characteristics of the study population (at least information on age, gender, spectrum of presenting symptoms).	10
	16	The number of participants satisfying the criteria for inclusion who did or did not undergo the index tests and/or the reference standard; describe why participants failed to undergo either test (a flow diagram is strongly recommended). -A bio-bank was used.	-
Test results	17	Time-interval between the index tests and the reference standard, and any treatment administered in between.	5
	18	Distribution of severity of disease (define criteria) in those with the target condition; other diagnoses in participants without the target condition.	4
	19	A cross tabulation of the results of the index tests (including indeterminate and missing results) by the results of the reference standard; for continuous results, the distribution of the test results by the results of the reference standard.	6
	20	Any adverse events from performing the index tests or the reference standard. -A bio-bank was used.	-
Estimates	21	Estimates of diagnostic accuracy and measures of statistical uncertainty (e.g. 95% confidence intervals).	13
	22	How indeterminate results, missing data and outliers of the index tests were handled.	5
	23	Estimates of variability of diagnostic accuracy between subgroups of participants, readers or centers, if done.	5
	24	Estimates of test reproducibility, if done.	5
DISCUSSION	25	Discuss the clinical applicability of the study findings.	7-9

Loss of epithelial membrane protein-2 expression confers an independent prognosticator in nasopharyngeal carcinoma: a cohort study

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Complete List of Authors:	Chen, Yi-Hsien; Department of Emergency Medicine, Chi-Mei Medical Center Wu, Li-Ching; Department of Pathology, Chi-Mei Medical Center Wu, Wen-Ren; Institute of Biomedical Science, National Sun Yat-sen University Lin, Hung-Jung; Department of Emergency Medicine, Chi-Mei Medical Center Lee, Sung-Wei; Department of Radiation Oncology, Chi-Mei Medical Center Lin, Ching-Yih; Deparment of Otolaryngology, Chi-Mei Medical Center Chow, Nan-Haw; Department of Pathology, National Cheng Kung University Hospital Huang, Hsuan-Ying; Department of Pathology, Chang Gung Memorial Hospital-Kaohsiung Medical Center, Chang Gung University Li, Chien-Feng; Department of Pathology, Chi-Mei Medical Center Shiue, Yow-Ling; National Sun Yat-sen University, Institute of Biomedical Science
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Loss of epithelial membrane protein-2 expression confers an independent prognosticator in nasopharyngeal carcinoma: a cohort study

Yi-Hsien Chen, [1] Li-Ching Wu, [2] Wen-Ren Wu, [3] Hung-Jung Lin, [1] Sung-Wei Lee, [4] Ching-Yih Lin,[5] Shih-Lun Chang, [6] Nan-Haw Chow, [7,8] Hsuan-Ying Huang, [9] Chien-Feng Li, [2,3,10,11] Han-Ping Hsu, [12] Yow-Ling Shiue [3,*]

[1] Department of Emergency Medicine, Chi-Mei Medical Center, Tainan, Taiwan
[2] Department of Pathology, Chi-Mei Medical Center, Tainan, Taiwan
[3] Institute of Biomedical Science, National Sun Yat-sen University, Kaohsiung, Taiwan
[4] Department of Radiation Oncology, Chi-Mei Medical Center, Liouying, Tainan, Taiwan
[5] Division of Gastroenterology and Hepatology, Department of Internal Medicine, Chi-Mei Medical Center, Tainan, Taiwan
[6] Department of Otolaryngology, Chi-Mei Medical Center, Tainan, Taiwan
[7] Department of Pathology, National Cheng Kung University Hospital, Tainan, Taiwan
[8] Institute of Basic Medical Sciences, National Cheng Kung University, Tainan, Taiwan
[9] Department of Pathology, Chang Gung Memorial Hospital-Kaohsiung Medical Center, College of Medicine, Chang Gung University, Kaohsiung, Taiwan
[10] National Institute of Cancer Research, National Health Heath Research Institute, Tainan, Taiwan
[11] Department of Biotechnology, Southern Taiwan University, Tainan, Taiwan
[12] College of Medicine, China Medical University, Taichung, Taiwan

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Correspondence to: Yow-Ling Shiue, Institute of Biomedical Science, National Sun Yat-sen University, Kaohsiung, Taiwan. E-mail: ylshiue@mail.nsysu.edu.tw

ABSTRACT

Objective: To evaluate the expression of epithelial membrane protein 2 (EMP2) protein and its clinicopathological associations in patients with nasopharyngeal carcinoma (NPC).

Design: Retrospective population-based cohort study.

Setting: This study was based on a biobank in Chi-Mei Medical Center (Tainan, Taiwan) from 1993 to 2002.

Participants: Biopsies of 124 consecutive NPC patients without initial distant metastasis and treated with consistent guidelines were assessed. Immunoexpressions of EMP2 were analyzed and the outcomes were correlated with clinicopathological features and patient survivals.

Results: Loss of EMP2 expression (49.2%) was correlated with advanced primary tumor (p=0.044), nodal status (p=0.045) and the 7th American Joint Committee on Cancer (AJCC) stage (p=0.027). In multivariate analyses, loss of EMP2 expression emerged as an independent prognosticator for worse disease-specific survival (DSS; p=0.015) and local recurrence-free survival (LRFS; p=0.030), along with AJCC stage III-IV (p=0.034, DSS; p=0.023, LRFS).

Conclusion: Loss of EMP2 expression is common and associated with adverse prognosticators, and might confer tumor aggressiveness through hampering its interaction with specific membrane protein(s) and hence, the downstream signal transduction pathway(s).

Introduction

Nasopharyngeal carcinoma (NPC) is an endemic head and neck epithelial malignancy in Southeastern Asia and Taiwan; strongly linked to Epstein-Barr virus (EBV).[1 2] The latter association is especially authentic for the differentiated and undifferentiated non-keratinizing carcinoma types, according to current World Health Organization tumor classification, although genetic and environmental factors also play certain roles in pathogenesis.[1-3] The advances in diagnostic imaging, radiation therapy, and adjuvant chemotherapy of NPC have achieved better locoregional control, while it appears less satisfactory in final treatment outcomes.[4 5] Even though being an important parameter, TNM staging still has space to improve in terms of providing the optimal prognostication to the patients. [1 4-6] Therefore, to identify potential biomarkers with better correlation to tumor growth and/or treatment outcomes in patients with NPC, subsequently, to aid in risk stratification and perhaps development of therapeutic targets, are indispensable.

Human epithelial membrane protein-2 gene (*EMP2*), mapped to chromosome 16, is highly conserved across vertebrates.[7-9] The expression pattern of *EMP2* partially overlaps to that of the peripheral myelin protein 22 (*PMP22*, also known as the growth arrest-specific-3, *GAS3*) transcript. By containing the claudin domain and sharing approximately 40% amino acid identity with PMP22/GAS3,[10] the EMP2 protein was detected as a novel member of this four-transmembrane (tetraspan) superfamily.[11] In humans, EMP2 has a discrete cell type and tissue distribution, with high levels observed in the lung and moderate levels in the eye, heart, thyroid, uterus and intestine. [10 12] Functionally, the best understood tetraspan proteins are connexins, which form the major structural element of gap junctions. Connexins play important roles in the regulation of cell growth and differentiation. Cancer cells usually have downregulated levels of gap junctions, and several lines of evidence suggest that loss of gap junctional intercellular communication is an important step in carcinogenesis. Reexpression

of connexins in cancer cells causes normalization of cell growth control and reduced tumor growth.[14 15] Accordingly, we aimed to systematically analyze EMP2 immunoexpression in patients with NPC and identified that loss of EMP2 expression is associated with adverse prognosticators, conferring to poor survivals.

MATERIALS AND METHODS

Patients and tumor specimens

The institutional review board approved the study by using formalin-fixed tissue of NPC for this study (IRB100-09-003). Available paraffin-embedded tissue blocks were retrieved from 124 NPC patients who underwent biopsy between Jan 1993 and Dec 2002. These patients were free of distant metastasis at initial presentation. The histological subtypes were reappraised according to the current World Health Organization classification and, the tumor staging was reevaluated with the 7th American Joint Committee on Cancer (AJCC) system by two pathologists, independently.

Immunohistochemical staining and assessment of EMP2 expression

Tissue sections of 3-µm thickness were cut onto precoated slides from paraffin-embedded tissue blocks and were next routinely deparaffinized with xylene and rehydrated with ethanol washes. Slides were heated by the microwave in a 10 mM citrate buffer (pH 6.0) for 7 min to retrieve antigens. Endogenous peroxidase was blocked with 3% H_2O_2. Slides were next washed by Tris-buffered saline for 15 min and subsequently incubated with a rabbit polyclonal primary antibody targeting EMP2 (Atlas Antibodies, Stockholm, Sweden) at a dilution of 1:75 for 1 h. Primary antibodies were detected using the DAKO ChemMate EnVision Kit (K5001, Carpinteria, CA, USA). The slides were incubated and developed with the secondary antibody for 30 min, and 3,3-diaminobenzidine for 5 min, followed by counterstained using Gill's Hematoxylin. Immunoexpression of EMP2 was scored by two

pathologists (CF Li and HY Huang) using a multiheaded microscope to reach a consensus for each case without prior knowledge of clinical and follow-up information. The percentage of tumor cells with EMP2 immunoexpression was recorded for each specimen and loss of EMP2 expression (negative) was defined in cases with staining ≤ 5% tumor cells (see Statistical analysis).

Treatment and follow-up

All 124 patients with follow-up for outcome have received complete course of radiotherapy (RT, total dose≥7,000 cGy) and also cisplatin-based chemotherapy in those of stage II-IV diseases, based on the previously published protocol.[16] The method of RT was in general uniform within this period. All patients were regularly monitored after RT until death or their last appointment with the mean follow-up duration being 59.6 months (range: 4-117).

Statistical analysis

Statistics were performed using SPSS 14.0 software (SPSS Inc., Chicago, IL, USA). Chi-square test was used to compare the EMP2 expression status and various clinicopathological parameters. The endpoints analyzed were disease-specific survival (DSS) and local recurrence-free survival (LRFS), calculated from the starting date of RT to the date of event developed. Patients lost to follow-up were censored on the latest follow-up date. Survival curves were plotted using the Kaplan-Meier method, and the log-rank test was performed to evaluate prognostic differences between groups. Multivariate analysis was carried out by the Cox proportional hazards model. However, as a component factor of the AJCC stage, primary tumor (T) and nodal status (N) was not introduced in multivariate comparisons. After testing a series of cutoff values in 5% increment, EMP2 expression was construed as negative when the expression index was ≤5% tumor cells. For all analyses, two-sided tests of significance were used with $p<0.05$ considered significant.

RESULTS

Immunohistochemical expression of EMP2 and associations with clinicopathological variables in NPC specimens

As shown in Table 1, 124 cases of NPC consisted of five keratinizing squamous cell carcinomas, 54 non-keratinizing differentiated carcinomas, and 65 non-keratinizing, undifferentiated carcinomas. A total of 95 males and 29 females with a mean age of 48.6 years (range, 20-83) included. Seven cases were classified as stage I, 31 as Stage II, 46 as Stage III, and 40 as Stage IV. Immunoexpression of EMP2 was observed and successfully scored in all cases. Tumor-adjacent normal respiratory epithelium (Figure 1A) or non-tumor epithelium with squamous metaplasia (Figure 1B) could be appreciated in 71 samples and all showed intense EMP2 immunoexpression. A wide range of stained tumor cell, characterized by cytoplasmic and/or membranous staining, varying from 0-90% (median, 30%) were detected in tumor elements. Of these, 63 cases showed characteristic EMP2 staining (>5% tumor cells; Figure 1C), while 61 cases were less than 5% staining and therefore classified as EMP2 negative (Figure 1D). Loss of EMP2 expression was significantly associated with cases featuring increment of primary tumor (p=0.004), nodal status (p=0.045) and AJCC stage (p=0.027) (Table 2). However, no significant association between the EMP2 expression level and other clinicopathological factor was found.

Prognostic impact of EMP2 expression in NPC

Patients with NPC more frequently progressed to disease-specific mortality with N2-N3 nodal status (p=0.002) and stage III-IV (p=0.007) (Table 3). Besides, patients with advanced AJCC stage III-IV held shorter DSS (p=0.07; Figure 2A) and LRFS (p=0.06; Figure 2B). The development of local recurrence was significantly associated with T3-T4 (p=0.027), N2-N3 status (p=0.023) and AJCC stage III-IV (p=0.005) with a medium duration of 24 months

(Table 3). Of note, EMP2 negative correlated to a more aggressive clinical course with a significantly shorter DSS (p=0.002; Figure 2C) and LRFS (p=0.005; Figure 2D) in patients with NPC. In multivariate analysis (Table 4), loss of EMP2 expression steady remained as a robust prognosticator for both inferior DSS [p=0.015, hazard ratio (HR)=1.969] and worse LRFS (p=0.030, HR=2.136), following tumor stage (p=0.034, HR=2.115; p=0.023, HR=3.046, for DSS and LRFS, respectively).

DISCUSSION

Loss of EMP2 immunostaining as one potent prognosticator for both DSS and LRFS in a subset of patients with NPC was sustained in this study. Intriguingly, we have also identified a significant association between loss of EMP2 expression and the overexpression of latent membrane protein 1 (p=0.007, data not shown), an important oncoprotein of EBV,[17] suggesting a potential role of EMP2 loss in EBV-associated tumor progression. However, significantly high EMP2 expression was found in ovarian cancer through activation of caveolins/glycosylphosphatidyl inositol-linked proteins,[18 19] and was identified as an early predictor of endometrial cancers with unfavorable outcome.[20 21] Due to non-neoplastic peritoneal surface tissues were complete negative for EMP2 staining, thus EMP2 was regarded as increased expression in tumor cells in ovarian cancer.[20] Moderately intense, diffuse immunohistochemical stainings of tumor cell cytoplasm were identified in endometrioid adenocarcinoma, serous carcinoma, mixed endometrioid and serous carcinoma, mixed endometrioid and clear cell carcinoma.[20] On the other hand, compared to undifferentiated ones, predominant expressions of EMP2 in cytoplasm and/or membrane of squamous metaplasias and non-keratinizing NPCs were found in our study, suggesting that loss of EMP2 expression might change its interactions with some membrane proteins in NPC. Surface expression of the α6β1 integrin was specifically increased by EMP2 in NIH3T3

fibroblasts.[21] Moreover, surface expression and trafficking of integrin αvβ3 during the window of implantation, which are essential for endometrial-blastocyst interaction in mice, were affected by the EMP2 level and the association between EMP2 and focal adhesion kinase.[20 23 24] In mammals, 18 α and eight β subunits assemble into 24 different integrins, which bind collagens, laminins, or arginine-glycine-aspartic acid-containing proteins. Integrins are regulated by conformational changes, clustering and trafficking, and regulatory mechanisms differ strongly between individual integrins and between cell types. Defective integrin activation or integrin signaling is associated with an array of pathological conditions.[25] Endocytosis and recycling are crucial in the regulation of integrin turnover and redistribution in adherent cells, especially during dynamic processes such as migration and invasion.[26] Therefore, EMP2 probably plays a tumor suppressor role through interacting with specific integrin(s) in epithelial cells and thereafter, manages regular signaling transduction in benign conditions.

In keep with the above finding, we uncovered that ectopic expression of *EMP2* in a malignant human urothelial cell line, J82, significantly reduced cell proliferation, cell cycle progression, migration and invasion in vitro (unpublished). Consistently, suppression subtractive hybridization technologies isolated mouse ortholog *Emp2*, which suppresses B-cell lymphoma tumorigenicity through a functional tumor suppressor phenotype.[9] The susceptibility to allogeneic cytotoxic T lymphocytes of a mouse malignant, *Emp2*-deficient cell line (MV)[9] has been enhanced by retroviral overexpression of *Emp2* gene.[13] Constitutive overexpression of EMP2 or other epithelial membrane proteins including EMP1, EMP3 and PMP22, in human HEK293 epithelial cells, leading to the development of apoptotic phenotypes, were demonstrated by purinergic receptor P2X, ligand-gated ion channel, 7 (P2RX7)-mediated cell blebbing, annexin V binding to plasma membrane, and cell death, through a caspase-dependent pathway. Physically, the C-terminal domain of P2RX7

protein associates with EMPs and mediates some aspects of the downstream signaling following P2RX7 activation.[27] All of these studies supported our clinical observations, reinforcing that EMP2 might play distinct characteristics in different cellular contexts. Indeed, the etiology of NPC is complex, including a host of viral, genetic and environmental factors.[14 17 28 29] In spite of cure for the majority of the patients, challenges still exist in the prevention of disease relapse and treatment of patients with refractory or metastatic NPC.[30-32] Therefore, for the first time, loss of EMP2 expression was identified as a biomarker independently correlated with tumor aggression to facilitate appropriate allocation of adjuvant therapy, suggesting its significance for patient-tailored strategy to manage high-risk NPCs.

Except for loss of EMP2 expression, significantly increased hazard ratios of DSS and LRFS in NPC patients with higher stages (III-IV) were further ascertained, analogous to other studies.[33-35] Additionally, we revealed significant correlations between loss of EMP2 expression and primary tumor, nodal status and stage in NPCs, indicating its prospective role in preventing NPC progression and aggressiveness. Although the precise characteristics of the EMP2 protein in NPC progression remain to be elucidated, the potential utility of EMP2 immunostaining as a prognostic biomarker in NPCs is assured.

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Table 1. Clinical pathological features of 124 nasopharyngeal carcinomas

Variable	*n* (%)
Gender	
Male	95 (76.6)
Female	29 (23.4)
Age (years)	
<60	98 (79.0)
≥60	26 (21.0)
Primary tumor (T)	
T1	30 (24.2)
T2	50 (40.3)
T3	21 (16.9)
T4	23 (18.5)
Nodal status (N)	
N0	24 (19.4)
N1	32 (25.8)
N2	48 (38.7)
N3	20 (16.1)
Stage	
I	7 (5.6)
II	31 (25.0)
III	46 (37.1)
IV	40 (32.2)
Histological grade	
Keratinizing	5 (4.0)
Non-keratinizing/differentiated	54 (43.5)
Non-keratinizing/undifferentiated	65 (52.4)
EMP2 expression level	
Positive (>5% tumor cells)	63 (50.8)
Negative (≤5% tumor cells)	61 (49.2)

Table 2. Expression level of EMP2 and correlations with clinicopathologic variables (*n*=124)

Variable	EMP2 expression score		*p*-value
	Positive (>5% tumor cells)	Negative (≤5% tumor cells)	
Gender			0.926
Male	43	52	
Female	20	9	
Age (years)			0.926
<60	50	48	
≥60	13	13	
Primary tumor (T)			0.044*
T1-T2	46	34	
T3-T4	17	27	
Nodal status (N)			0.045*
N0-N1	34	22	
N2-N3	29	39	
Stage			0.027*
I-II	25	13	
III-IV	38	48	
Histological grade			0.879
Keratinizing	3	2	
Non-keratinizing/differentiated	28	26	
Non-keratinizing/undifferentiated	32	33	

*, Statistically significant

Table 3. Univariate log-rank analysis of EMP2 expression score on survival outcome (*n*=124)

Variable	*n*	DSS [1]		LRFS [2]	
		n	*p*-value	*n*	*p*-value
Gender			0.878		0.346
Male	95	45		30	
Female	29	14		7	
Age (years)			0.996		0.755
<60	98	48		29	
≥60	26	11		8	
Primary tumor (T)			0.065		0.027*
T1-T2	80	32		19	
T3-T4	44	27		18	
Nodal status (N)			0.002*		0.023*
N0-N1	56	18		12	
N2-N3	68	41		25	
Stage			0.007*		0.005*
I-II	38	10		3	
III-IV	86	49		32	
Histological grade			0.157		0.900
Keratinizing/Non-keratinizing	47	40		15	
Undifferentiated	77	39		22	
EMP2 expression level			0.002*		0.005*
Positive (>5% tumor cells)	63	21		13	
Negative (≤5% tumor cells)	61	38		24	

*, Statistically significant; [1] DSS, disease-specific survival; [2] LRFS, local recurrence-free survival

Table 4. Multivariate survival analysis of EMP2 expression level on survival outcome

Variable	DSS [1]		LRFS [2]	
	HR[3] (95% CI[4])	*p*-value	HR[3] (95% CI[4])	*p*-value
AJCC Stage		0.034*		0.023*
I-II	1		1	
III-IV	2.115 (1.057-4.232)		3.046 (1.171-7.919)	
EMP2 expression level		0.015*		0.030*
Positive (>5% tumor cells)	1		1	
Negative (≤5% tumor cells)	1.969 (1.144-3.391)		2.136 (1.076-4.237)	

*, Statistically significant; [1] DSS, disease-specific survival; [2] LRFS, local recurrence-free survival; [3] HR, hazard ratio; [4] CI, confidence interval

Figure legends

Figure 1 Immunohistochemically, non-tumor respiratory epithelium (A) and those with squamous metaplasia (B), demonstrate diffuse and strong EMP2 immunoexpression, which can also be appreciated in representative non-keratinizing carcinoma (C) but not in undifferentiated one (D).

Figure 2 Kaplan-Meier plotting illustrates the prognostic significance of tumor stage for (A) disease-specific survival (DSS) and (B) local recurrence-free survival (LRFS), respectively. The predictive value of EMP2 expression is also demonstrated (C, D).

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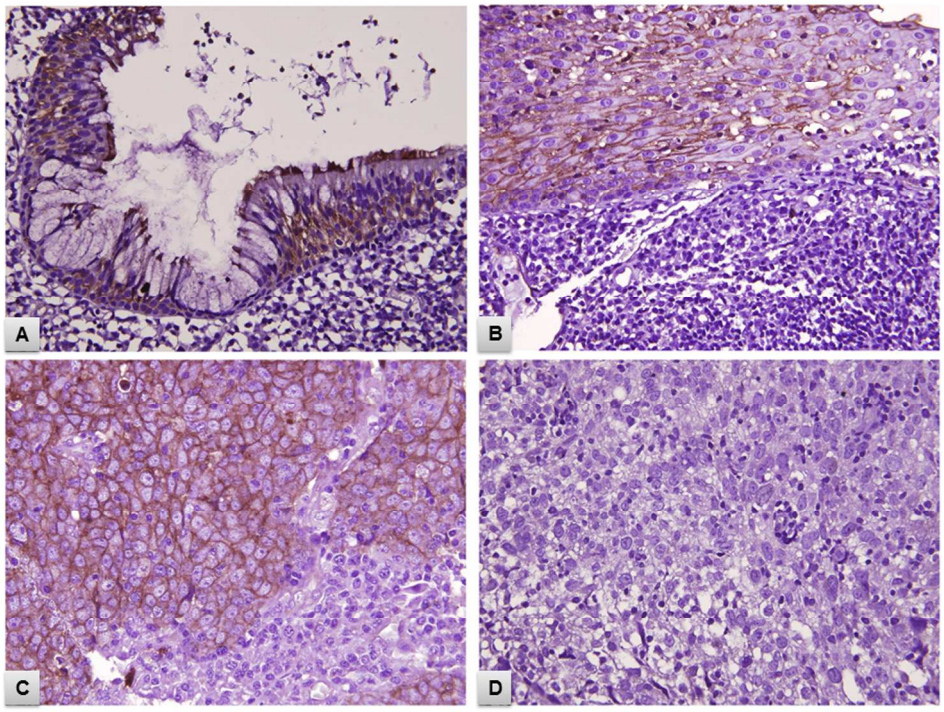

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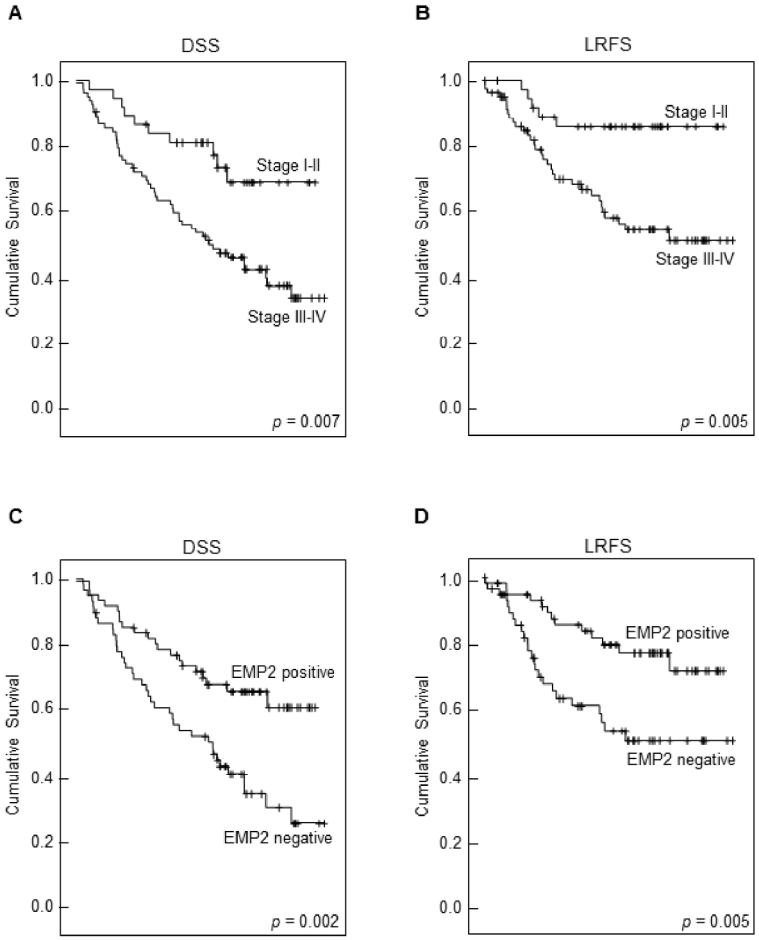

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(version January 2003)

Section and Topic	Item #		On page #
TITLE/ABSTRACT/ KEYWORDS	1	Identify the article as a study of diagnostic accuracy (recommend MeSH heading 'sensitivity and specificity').	1/2/1
INTRODUCTION	2	State the research questions or study aims, such as estimating diagnostic accuracy or comparing accuracy between tests or across participant groups.	3-4
METHODS			
Participants	3	The study population: The inclusion and exclusion criteria, setting and locations where data were collected.	4
	4	Participant recruitment: Was recruitment based on presenting symptoms, results from previous tests, or the fact that the participants had received the index tests or the reference standard?	4
	5	Participant sampling: Was the study population a consecutive series of participants defined by the selection criteria in item 3 and 4? If not, specify how participants were further selected.	4
	6	Data collection: Was data collection planned before the index test and reference standard were performed (prospective study) or after (retrospective study)?	2
Test methods	7	The reference standard and its rationale.	4
	8	Technical specifications of material and methods involved including how and when measurements were taken, and/or cite references for index tests and reference standard.	4-5
	9	Definition of and rationale for the units, cut-offs and/or categories of the results of the index tests and the reference standard.	5
	10	The number, training and expertise of the persons executing and reading the index tests and the reference standard.	5
	11	Whether or not the readers of the index tests and reference standard were blind (masked) to the results of the other test and describe any other clinical information available to the readers.	5
Statistical methods	12	Methods for calculating or comparing measures of diagnostic accuracy, and the statistical methods used to quantify uncertainty (e.g. 95% confidence intervals).	5
	13	Methods for calculating test reproducibility, if done.	5
RESULTS			
Participants	14	When study was performed, including beginning and end dates of recruitment.	4
	15	Clinical and demographic characteristics of the study population (at least information on age, gender, spectrum of presenting symptoms).	10
	16	The number of participants satisfying the criteria for inclusion who did or did not undergo the index tests and/or the reference standard; describe why participants failed to undergo either test (a flow diagram is strongly recommended). -A bio-bank was used.	-
Test results	17	Time-interval between the index tests and the reference standard, and any treatment administered in between.	5
	18	Distribution of severity of disease (define criteria) in those with the target condition; other diagnoses in participants without the target condition.	4
	19	A cross tabulation of the results of the index tests (including indeterminate and missing results) by the results of the reference standard; for continuous results, the distribution of the test results by the results of the reference standard.	6
	20	Any adverse events from performing the index tests or the reference standard. -A bio-bank was used.	-
Estimates	21	Estimates of diagnostic accuracy and measures of statistical uncertainty (e.g. 95% confidence intervals).	13
	22	How indeterminate results, missing data and outliers of the index tests were handled.	5
	23	Estimates of variability of diagnostic accuracy between subgroups of participants, readers or centers, if done.	5
	24	Estimates of test reproducibility, if done.	5
DISCUSSION	25	Discuss the clinical applicability of the study findings.	7-9