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

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

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 nasopharyngeal carcinoma patients without initial distant metastasis and treated with consistent guidelines were assessed. Immunoexpressions of EMP2 were analysed and the outcomes were correlated with clinicopathological features and patient survivals.

Primary and secondary outcome measures: 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 tumour (p = 0.044), nodal status (p = 0.045) and the 7th American Joint Committee on Cancer 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 the American Joint Committee on Cancer stages 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 tumour 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).1–3 The latter association is especially authentic for the differentiated and undifferentiated non-keratinising carcinoma types, according to current WHO tumour classification, although genetic and environmental factors also play certain roles in pathogenesis.1,2,4 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.5,6 Even though being an important parameter, Tumour, Node, Metastasis staging still has space to improve in terms of providing the optimal prognostication to the patients.1,5,7 Therefore, to identify potential biomarkers with better correlation to tumour growth and/or treatment outcomes in patients with NPC, subsequently, to aid in risk stratification and perhaps development of therapeutic targets, are indispensable.

ARTICLE SUMMARY
Article focus
- Recent studies have suggested that EMP2 plays a tumour suppressor role in B cell lymphomas.
- Immunoexpression of EMP2 was retrospectively assessed in biopsies of 124 consecutive patients with nasopharyngeal carcinoma.

Key messages
- Loss of EMP2 expression significantly correlates with advanced primary tumour, nodal status and AJCC stage.
- In multivariate analyses, loss of EMP2 expression emerges as an independent prognosticator for worse disease-specific survival and local recurrence-free survival.

Strengths and limitations of this study
- Significant correlation between loss of EMP2 expression and several clinicopathologic variables supported its potential role in nasopharyngeal carcinomas.
- The molecular mechanisms underlying EMP2 action require to be elucidated.
Human epithelial membrane protein-2 gene (EMP2), mapped to chromosome 16, is highly conserved across vertebrates. 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, EMP2 was detected as a novel member of this four-transmembrane (tetraspan) superfamily. 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. 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. Re-expression of connexins in cancer causes normalisation of cell growth control and reduced tumour growth. Accordingly, we aimed to systematically analyse EMP2 immunoeexpression 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 tumour 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 January 1993 and December 2002. These patients were free of distant metastasis at initial presentation. The histological subtypes were reappraised according to the current WHO classification and, the tumour staging was re-evaluated 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% H2O2. 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, California, 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 haematoxylin. Immunoexpression of EMP2 was scored by two pathologists (C-FL and HYH) using a multiheaded microscope to reach a consensus for each case prior knowledge of clinical and follow-up information. The percentage of tumour cells with EMP2 immunoexpression was recorded for each specimen and loss of EMP2 expression (negative) was defined in cases with staining ≤5% tumour cells (see the Statistical analysis section).

Treatment and follow-up

All 124 patients with follow-up for outcome have received complete course of radiotherapy (RT, total dose ≥7000 cGy) and also cisplatin-based chemotherapy in those of stage II–IV diseases, based on the previously published protocol. 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 V.14.0 software (SPSS Inc). \chi^2 Test was used to compare the EMP2 expression status and various clinicopathological parameters. The end points analysed were disease-specific survival (DSS) and local recurrence-free survival (LRFS), calculated from the starting date of RT to the

| Variable | n (%) |
|----------|-------|
| Gender   |       |
| Male     | 95 (76.6) |
| Female   | 29 (23.4) |
| Age (years) |       |
| <60      | 98 (79.0) |
| ≥60      | 26 (21.0) |
| Primary tumour (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 |       |
| Keratinising | 5 (4.0) |
| Non-keratinising/differentiated | 54 (43.5) |
| Non-keratinising/undifferentiated | 65 (52.4) |
| EMP2 expression level |       |
| Positive (≥5% tumour cells) | 63 (50.8) |
| Negative (≤5% tumour cells) | 61 (49.2) |
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 tumour (T) and nodal status (N) was not
introduced in multivariate comparisons. After testing
a series of cut-off values in 5% increment, EMP2
expression was construed as negative when the expres-
sion index was \( \leq 5\% \) tumour 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
keratinising squamous cell carcinomas, 54 non-kerati-
iscing differentiated carcinomas and 65 non-keratinising
undifferentiated carcinomas. A total of 95 men and 29
women with a mean age of 48.6 years (range, 20–83)
were included. Seven cases were classified as stage I, 31
as stage II, 46 as stage III and 40 as stage IV. Immunohistochemical
expression of EMP2 was observed and successfully
scored in all cases. Tumour-adjacent normal respiratory
epithelium (figure 1A) or non-tumour epithelium with
squamous metaplasia (figure 1B) could be appreciated
in 71 samples and all showed intense EMP2 immunoexpression.
A wide range of stained tumour cell,
characterised by cytoplasmic and/or membranous
staining, varying from 0% to 90% (median, 30%)
were detected in tumour elements. Of these, 63 cases
showed characteristic EMP2 staining (>5% tumour cells;
figure 1C), while 61 cases were <5% staining and
therefore classified as EMP2 negative (figure 1D). Loss
of EMP2 expression was significantly associated with
cases featuring increment of primary tumour (\( p = 0.004 \)),
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 stages III–IV \( (p = 0.007) \) (table 3).
Besides, patients with advanced AJCC stages 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 stages 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 \( (p = 0.015, \) HR=1.969) and worse
LRFS \( (p = 0.030, \) HR=2.136), following tumour 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 progno-
siticator for both DSS and LRFS in a subset of patients
with NPC was sustained in this study. Intriguingly, we

Figure 1  Immunohistochemically
non-tumour respiratory epithelium
(A) and those with squamous
metaplasia (B) demonstrate
diffuse and strong EMP2
immunoexpression, which can
also be appreciated in
representative non-keratinising
carcinoma (C) but not in
undifferentiated one (D).
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, suggesting a potential role of EMP2 loss in EBV-associated tumour progression. However, significantly high EMP2 expression was found in ovarian cancer through activation of caveolins/glycosylphosphatidylinositol-linked proteins and was identified as an early predictor of endometrial cancers with unfavourable outcome. Due to non-neoplastic

### Table 2

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

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

*Statistically significant.

### Table 3

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

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

*Statistically significant.

DSS, disease-specific survival; LRFS, local recurrence-free survival.
peritoneal, surface tissues were complete negative for EMP2 staining, thus EMP2 was regarded as increased expression in tumour cells in ovarian cancer. Moderate intense diffuse immunohistochemical stainings of tumour cell cytoplasm were identified in endometrioid adenocarcinoma, serous carcinoma, mixed endometrioid and serous carcinoma, mixed endometrioid and clear cell carcinoma. On the other hand, compared with undifferentiated ones, predominant expressions of EMP2 in cytoplasm and/or membrane of squamous metaplasias and non-keratinising 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. Moreover, surface expression and trafficking of integrin

| Table 4  | Multivariate survival analysis of EMP2 expression level on survival outcome |
|----------|--------------------------------------------------------------------------|
| **Variable** | **DSS** | **LRFS** |
|          | **HR (95% CI)** | **p Value** | **HR (95% CI)** | **p Value** |
| AJCC stage | | | | |
| I–II | 1 | 0.034* | 1 | 0.023* |
| III–IV | 2.115 (1.057 to 4.232) | | 3.046 (1.171 to 7.919) | |
| EMP2 expression level | | | | |
| Positive (>5% tumour cells) | 1 | 0.015* | 1 | 0.030* |
| Negative (≤5% tumour cells) | 1.969 (1.144 to 3.391) | | 2.136 (1.076 to 4.237) | |

*Statistically significant.
AJCC, American Joint Committee on Cancer; DSS, disease-specific survival; LRFS, local recurrence-free survival.
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.26 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 signalling 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 tumour suppressor role through interacting with specific integrin(s) in epithelial cells and, thereafter, manages regular signalling transduction in benign conditions.

In addition to 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 hybridisation technologies isolated mouse orthologue Emp2, which suppresses B cell lymphoma tumorigenicity through a functional tumour suppressor phenotype.10

The susceptibility to allogeneic cytotoxic T lymphocytes of a mouse malignant, Emp2-deficient cell line (MV)10 has been enhanced by retroviral overexpression of Emp2 gene.27 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 signalling following P2RX7 activation.28 All these studies supported our clinical observations, reinforcing that EMP2 might play distinct characteristics in different cellular contexts. Indeed, the aetiology of NPC is complex, including a host of viral, genetic and environmental factors.3 29 30 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.31—33 Therefore, for the first time, loss of EMP2 expression was identified as a biomarker independently correlated with tumour 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 HRs of DSS and LRFS in NPC patients with higher stages (III–IV) were further ascertained, analogous to other studies.34—36 Additionally, we revealed significant correlations between loss of EMP2 expression and primary tumour, 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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Funding This work was supported by grants DOH99-TD-C-111-004 (Department of Health, Taiwan) to C-FL for tissue dissection and immunohistochemical analysis; CMFHR10119 (Chi-Mei Medical Center) to L-CW for case history analysis; 98-2311-B-110-001-MY3 (National Science Council, Taiwan) to Y-LS for target (EMP2) prioritisation.

Competing interests None.

Ethics approval Ethics approval was provide by the institutional review board (IRB100-09-003).

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement The original immunostaining and statistical data are available from the corresponding author at yshiue@mail.nsysu.edu.tw.

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