PEER REVIEW HISTORY

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ARTICLE DETAILS

TITLE (PROVISIONAL)	Loss of epithelial membrane protein-2 expression confers an independent prognosticator in nasopharyngeal carcinoma: a cohort study
AUTHORS	Yi-Hsien Chen, Li-Ching Wu, Wen-Ren Wu, Hung-Jung Lin, Sung-Wei Lee, Ching-Yih Lin, Shih-Lun Chang, Nan-Haw Chow, Hsuan-Ying Huang, Chien-Feng Li, Han-Ping Hsu and Yow-Ling Shiue

VERSION 1 - REVIEW

REVIEWER	Chen-Yong Lin, Associate Professor of Biochemistry and Molecular Biology University of Maryland, Baltimore
REVIEW RETURNED	06/02/2012

GENERAL COMMENTS	This is a simple and concise manuscript that reveals the pathological importance of epithelial membrane protein-2 (EMP2) in nasopharyngeal carcinoma (NPC). The quality of immunohistochemical staining is excellent. The outcome of this study provides a clinically important prognostic biomarker for the disease. The writing is largely fine but some sentences are difficult to understand. In p.8, the three sentences from lines 193 through 202 need to be revised. In p.9, the sentence from line 209 to 212 could be rewritten into two for better clarity.

REVIEWER	Joseph S. Pagano, MD Lineberger Professor of Cancer Research and Director Emeritus Professor of Medicine and Microbiology and Immunology University of North Carolina at Chapel Hill Dr. Joseph Pagano has no competing interests.
REVIEW RETURNED	20/02/2012

GENERAL COMMENTS	The authors report an association between loss of expression of EMP2 protein, a member of the tetraspan superfamily, expressed in epithelial membranes, and poor prognosis in NPC. The results seem convincing and add a new prognostic marker to those already available, which is important in this ultimately lethal disease. However the authors do not consider the relative or possible unique value of EMP2 in comparison with other markers that have been evaluated for the same purpose and where mechanism has been better studied in the context of NPC. There is an extensive literature not cited here on the relation of numerous invasion, metastasis and angiogenic factors that contribute to the oncogenic processes at work in cellular proliferation and tumor progression of NPC, most

	linked to the principal EBV oncoprotein LMP1. Also this paper is devoid of EBV studies even detection of EBV EBERS. Better would be staining for LMP1 protein and correlation with EMP2 levels in NPC, which would add significantly to the value of this report. Other Comments: 1. What tests were done to confirm specificity of EMP2 antiserum used? 2. Is EMP2 expressed in normal nasopharyngeal tissue? 3. Has effect of loss of EMP2 been assessed in other malignancies?

VERSION 1 – AUTHOR RESPONSE

Re: Reviewer: Chen-Yong Lin,
Associate Professor of Biochemistry and Molecular Biology
University of Maryland, Baltimore

This is a simple and concise manuscript that reveals the pathological importance of epithelial membrane protein-2 (EMP2) in nasopharyngeal carcinoma (NPC). The quality of immunohistochemical staining is excellent. The outcome of this study provides a clinically important prognostic biomarker for the disease. The writing is largely fine but some sentences are difficult to understand. In p.8, the three sentences from lines 193 through 202 need to be revised. In p.9, the sentence from line 209 to 212 could be rewritten into two for better clarity.

In lines 193-202 (lines 197-205 in revision), we described information relating to how downregulation of EMP2 was selected as a potential biomarker. To improve the methodologies and statements clearer for readers, we have revised these sentences into:
¬ Line 197, 'suppression subtractive hybridization technologies isolated…'

¬ Lines 199-200, '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' has been revised into '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'

¬ Lines 201-205, '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.' has been revised into
'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.'

¬ Line 205, 'Physically' was added in the beginning of 'the C-terminal domain…'

Re: Reviewer: Joseph S. Pagano, MD
Lineberger Professor of Cancer Research and Director Emeritus
Professor of Medicine and Microbiology and Immunology

University of North Carolina at Chapel Hill

Dr. Joseph Pagano has no competing interests. The authors report an association between loss of expression of EMP2 protein, a member of the tetraspan superfamily, expressed in epithelial membranes, and poor prognosis in NPC. The results seem convincing and add a new prognostic marker to those already available, which is important in this ultimately lethal disease. However the authors do not consider the relative or possible unique value of EMP2 in comparison with other markers that have been evaluated for the same purpose and where mechanism has been better studied in the context of NPC.

¬ EMP2 is a novel molecule in the field of cancer research. It involves in focal adhesion kinase 1 (PTK2 or FAK)/v-src sarcoma viral oncogene homolog (SRC) (Fu et al. 2011; PLoS One 6:e19945) (lines 183) and caveolins/glycosylphosphatidyl inositol-linked proteins (Wadehra et al. 2004; Mol Biol Cell 15:2073-83) signaling pathways (lines 169-170); regulates the expression levels of $\alpha v\beta 3$ (Wadehra et al. 2005; Dev Biol 287:336-45) (lines 181-182) and $\alpha 6\beta 1$ (Wadehra et al. 2002; J Biol Chem 277:41094-100) (lines 180-181) integrins, and class I major histocompatibility complex proteins (Wadehr et al. 2003; Clin Immunol 107:129-36) (line 199-201). So far, only purinergic receptor P2X, ligand-gated ion channel, 7 (P2RX7) was identified to be directly interacted with EMP2 protein (Wilson et al. 2002; J Biol Chem 277:34017-23) (lines 205-206). Each of these proteins was identified to be a component of the plasma membrane. According to our observation of the subcellular location of EMP2, we discussed this in Lines 205-206.

¬ Among these proteins, EMP2 induces membrane blebbing by caspase-dependent pathway in HEK293 cells, and directly interacts with the C-terminus of P2RX7 (Wilson et al. 2002; J Biol Chem 277:34017-23) So far, there is no documentation connecting P2RX7 expression levels to NPCs. Therefore, a systematic study on EMP2 network in different NPC-derived cellular models will be a top priority to identify candidate/direct regulators or modulators. Further reconfirmation on patient specimens will be the subsequent strategy. We therefore, did not evaluate any other markers in tissue specimens at this moment.

There is an extensive literature not cited here on the relation of numerous invasion, metastasis and angiogenic factors that contribute to the oncogenic processes at work in cellular proliferation and tumor progression of NPC, most linked to the principal EBV oncoprotein LMP1. Also this paper is devoid of EBV studies even detection of EBV EBERS. Better would be staining for LMP1 protein and correlation with EMP2 levels in NPC, which would add significantly to the value of this report.

¬ We have additionally performed immunohistochemical analysis of latent membrane protein 1 (LMP1) on this cohort. We found a significant correlation between overexpression of LMP1 and loss of EMP2 expression (p=0.007; data not shown). This has been revised in the ‘Discussion’ section (lines 165-168).

Other Comments:
1. What tests were done to confirm specificity of EMP2 antiserum used?
2. Is EMP2 expressed in normal nasopharyngeal tissue?
3. Has effect of loss of EMP2 been assessed in other malignancies?

1. To confirm the specificity of EMP2 antiserum, we have conducted several tests (unpublished) to substantiate our study.
¬ Firstly, we examined endogenous EMP2 mRNA and protein expression levels by quantitative RT-PCR and immunoblotting analysis, in normal human urothelial cell (HUC), low (RT4), moderate (TSGH8301) and high (J82)-grade urothelial bladder carcinomas (UBC)-derived cells. Results demonstrated that EMP2 mRNA and protein levels are resemble in three cell lines, i.e., gradually

decreased in UBC cells with higher grades (TSGH8301 and J82).

¬ Secondly, we transfected the pEMP2-EGFP plasmid into the J82 cell line for 24, 48, 72, and 96 h, subjected to SDS/PAGE, and immunoblotted with anit-EMP2 antibody. A clear immunoblotting band with ~46 kDa, corresponded to the molecular weight of EMP2-GFP fusion protein was identified.

¬ Thirdly, we transfected two distinct shRNAi plasmids targeting EMP2 gene into the RT4 cell line for five days, quantitative RT-PCR and immunoblotting analysis displayed that both EMP2 mRNA and protein levels were significantly downregulated.

2. Regarding to EMP2 expression in normal nasopharyngeal tissue, we have addressed this in the 'RESULTS' section, '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.' (lines 139-141)

3. Indeed, the effect of loss of EMP2 was not assessed in other malignancies.
¬ However, gene transfer of EMP2 into MV cells (line 199-201) inhibited tumor formation, indicating that EMP2 acts as a functional tumor suppressor in B lymphoma (Wang et al. 2001; Blood 97:3890-3895).

¬ Furthermore, our studies on urothelial bladder carcinomas identified that EMP2 mRNA and immunohistostaining levels were significantly lower expressed in poorly-, compared to well-differentiated lesions. Also, EMP2 low expression significantly conferred to worse disease-specific and metastasis-free survivals (unpublished, will be submitted soon).