Prognostic significance of pre-treatment latent membrane protein 1 from nasopharyngeal swabs for stage III–IVA nasopharyngeal carcinoma

Q Guo1,2†, IWK Tham2†, S Lin1, Y Su3, Z Chen3, J Lin1, L Han1, Q Lin, MD1,4, J Pan1*, JJ Lu2*

Abstract

Background

Previous studies have proposed that the latent membrane protein 1 (LMP1) gene is related to the pathogenesis and progression of nasopharyngeal carcinoma. However, the role of pre-treatment LMP1 as a prognostic factor has not been fully addressed, and most previous studies did not utilize polymerase chain reaction detection techniques. We aimed to investigate whether the presence of pre-treatment LMP1 from nasopharyngeal swabs detected by polymerase chain reaction would allow prognostication and potentially treatment stratification prior to any intervention.

Methods

From June 2007 to June 2008, 87 patients with stage III–IVA nasopharyngeal carcinoma who completed radical radiation therapy were enrolled prospectively. All patients underwent nasopharyngeal swabs for LMP1 prior to any intervention.

Results

Of the 87 swab samples, LMP1 was detected in 75 (86.2%). Overall survival rates were significantly higher in patients without LMP1 expression (LMP1−) compared with those with LMP1 (LMP1+) (100% vs. 71.6%, respectively, P = 0.034). In addition, clear trends of improved regional relapse-free, distant metastasis-free and progression-free survival rates were noted in the LMP1− group compared with the LMP1+ group (P = 0.076, = 0.067 and P = 0.058, respectively). Local control between the two groups was similar (P = 0.165).

Conclusion

Pre-treatment LMP1 as detected by nasopharyngeal swabs using polymerase chain reaction is an adverse prognostic factor for stage III–IVA nasopharyngeal carcinoma and potentially can be used as a treatment stratification tool.

Background

Outcomes of early stage (stages I–II) nasopharyngeal cancer (NPC) with contemporary treatment have been satisfactory, with 5-year overall survival rates at 90% or greater. However, the prognosis of locoregionally advanced NPC can still be improved. While local control can be excellent even for large T3–4 tumours using intensity modulated radiation therapy, the rate of distant failure can approach 20%–25% at 5 years for stage III–IVB disease when optimally treated with concurrent chemoradiation therapy. Due to the poor tolerability of adjuvant chemotherapy, efforts are currently underway to evaluate the role of neo-adjuvant chemotherapy, in particular, to improve distant metastasis-free survival rates. Besides advanced clinical stage, there is currently no reliable prognostic marker to select patients at higher risk of distant metastases prior to onset of treatment.

Latent membrane protein 1 (LMP1) is one of the major transformation-related Epstein–Barr virus (EBV) encoded genes. It has been identified as encoding an oncoprotein that functions as a constitutively active tumour necrosis factor receptor (TNFR) and is thought to be a key modulator in NPC pathogenesis and progression, particularly in invasion and metastasis. However, the clinical significance of LMP1 has not been fully elucidated, with some studies reporting that it is not a prognostic factor or an adverse prognostic factor or a good prognostic marker. A recent meta-analysis of 12 such studies has suggested that LMP1 expression was correlated with an increased risk of distant metastases.

Conclusion

Late studies have demonstrated that the detection of LMP1 using PCR techniques may have a lower sensitivity than polymerase chain reaction (PCR) techniques used to detect DNA by techniques and are also invasive. Early studies have demonstrated that the detection of LMP1 using PCR detection in nasopharyngeal swab specimens is feasible in the setting of NPC screening. However, evaluation of pre-treatment LMP1 as a prognostic factor of locoregionally advanced NPC patients using this technique has not been reported. The
The purpose of this prospective study is to evaluate the role of the pre-treatment LMP1 status on the prognosis of locoregionally advanced NPC by PCR, a non-invasive and more accurate method, and aim to provide laboratory evidence for the risk grouping of patients.

**Materials and methods**

The study population consisted of 87 patients with newly diagnosed, pathologically confirmed poorly differentiated or undifferentiated NPC who received radical chemoradiation therapy between June 2007 and June 2008 in Fujian Provincial Cancer Hospital, China. All patients were clinically stage III to IVA (1992 Chinese staging system), had a Karnofsky performance status score of more than 70, and gave informed consent prior to study entry. Median follow-up time was 47 months (range, 10–59 months). Other patient characteristics are detailed in Table 1.

After topical administration of local anaesthesia (1% cocaine solution), a cotton swab on a 15-cm long stick would be inserted into the nasal cavity towards the nasopharyngeal wall. The cotton swab would then be pressed against the posterior and lateral nasopharyngeal walls and swept over the surface of the walls several times to obtain a DNA sample. On withdrawal, the cotton swab would immediately be immersed in phosphate buffer solution (PBS) and sent for processing. At the laboratory, a DNA kit (QIAGEN, Shanghai, China) was used to extract the DNA. The specimens would be immersed and washed in 2 mL of PBS to retrieve suspended cells. After centrifugation, the supernatant would be discarded, and 400 µL of water would be added to the pellet. A 400 µL aliquot of phenol/chloroform solution would be added and extracted twice, and 400 µL of chloroform would then be added and extracted once. After ethanol precipitation and centrifugation, the supernatant would be discarded, and the pellet then prepared for direct use in PCR by air drying and dissolution in 50 µL of water.

For PCR amplification, oligonucleotide primers (sense, BN1; antisense, BN2; see below) were used to detect the presence of LMP1 in extracted DNA. PCR amplification was performed in a total volume of 50 µL, consisting of 5 µL extracted DNA, 1 µL sense and anti-sense primers, 4 µL deoxynucleotide triphosphate mixture, 5 µL 10× PCR buffer (TaKaRa Biotechnology, Dalian, China) and 1.25 units of Taq DNA polymerase (TaKaRa Biotechnology, Dalian, China). Samples were amplified for 35 cycles using the following procedures: denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 60 seconds in a programmable thermal controller (Bio-Rad Laboratories, Shanghai, China) without the overlay of mineral oil. Products then were examined by 1.5% agarose gel electrophoresis in 1× Tris–boric acid–ethylenediamine tetraacetic acid solution and stained with ethidium bromide to determine the presence or absence of the special PCR product. The marker that was used in this study was the product from Shanghai Bohong Company. We chose to amplify regions of the EBV LMP1 gene (sense primer BN1, 5′-AGCGACTCTGCTGGAAATGAT-3′; anti-sense primer BN2, 5′-TGATTAGCTAA GGCATTCCCA-3′) for the identification of viral DNA. Negative

<table>
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<tr>
<th>Characteristics</th>
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<th>LMP1 negative (N = 12)</th>
<th>χ²</th>
<th>P value</th>
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<td>7 (58.33)</td>
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<td>10 (83.33)</td>
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<td>2 (16.67)</td>
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<td>7 (58.33)</td>
<td>0.000</td>
<td>1.000</td>
</tr>
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<td>CRT</td>
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<td>5 (41.67)</td>
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IMRT, intensity-modulated radiation therapy; CRT, conventional radiation therapy.

Table 1: Demographic and clinical characteristics by latent membrane protein 1 (LMP1) status.
control samples containing water were processed in parallel with all patient samples. DNA from the B95-8 cell line was used as the EBV positive control.

Two-dimensional conventional radiation therapy (2D-CRT) was utilized for 36 patients. Patients were simulated and treated supine with a customized head shell, in 1.8 or 2 Gy daily fractions, five fractions per week for 7–8 weeks. Phase 1 consisted of two large parallel-opposed lateral faciocervical fields treating to 36–40 Gy. The fields were then taken off-cord to 50 Gy, with bilateral posterior neck matching election fields. Phase 2 brought the nasopharynx dose to a total of 68–72 Gy using an anterior and two lateral fields. Node negative patients received 50–54 Gy to the neck, whereas node positive patients were boosted to 64–70 Gy at the involved nodal region. Thirty-six patients in our series received CRT, including 2 patients with T_{1,2} received routine brachytherapy boost after external beam radiation. The cumulative dose to the primary disease ranged from 69.2 to 75.25 Gy.

Three patients receiving 2D-CRT and eight receiving IMRT had a residual tumour after the completion of external beam radiation. A boost of 6–10 Gy was delivered using intracavitary brachytherapy, conventionally planned external beam radiation or IMRT. All 87 patients received neoadjuvant chemotherapy consisting of cisplatin (80 mg/m² divided in three daily doses) and paclitaxel (135 mg/m² on day 1) administered every 2 weeks for two cycles. Thirty-one patients received cisplatin-based concurrent chemotherapy for 1 or 2 cycles and 36 received adjuvant chemotherapy for 1–3 cycles. All patients were evaluated weekly during radiation therapy and were required to be followed-up after the completion of radiotherapy every 3 months in the first 2 years, every 6 months from year 2 through year 5, and annually thereafter. Each follow-up included a complete examination including flexible fiberoptic endoscopy, basic serum chemistry, chest X-ray and ultrasound of liver and abdomen. Either computed tomography or magnetic resonance imaging of the head and neck was performed after the completion of treatment and then after every 6 months.

Data were analysed using SPSS version 16.0 (SPSS Inc., Chicago, IL). Comparisons of the distributions of clinical stages and socio-demographic characteristics of different LMP1 expression groups were performed using a chi-square test. Overall survival (OS), progression-free survival (PFS), local control (LC), regional relapse-free survival (RRFS) and distant metastasis-free survival (DMFS) rates were calculated by the Kaplan–Meier method. The duration of OS was measured from diagnosis until death or date of the last follow-up visit for patients who were still alive. The time to progression, local failure, regional failure and distant metastasis was measured from the date of the diagnosis until documented treatment failure. The log-rank test was used to detect the significant difference in survivals between different LMP1 expression groups. Multivariate analysis using the Cox proportional hazard model was performed for the previously mentioned end-points to define independent predictors among various potential prognostic factors. A two-sided P value of ≤0.05 was considered statistically significant.

Results

To address potential selection bias, the distribution of demographic and disease characteristics (including gender, age, T-category, N-category, overall stage and radiation modality) between patients in this study (n = 87) and all other diagnosed NPC patients in our hospital with stage III–IVA (n = 223) in the same period were compared. No statistically significant difference was found between these groups of patients (data not shown). Of the 87 swab samples, LMP1 was detected in 75, with a positive predictive value of 86.2% (Figure 1). The two groups with different LMP1 expression were similar in terms of gender, age, T-category, N-category, clinical stage and radiation modality (Table 1).

The median follow-up time was 47 months (range, 10–59 months) for our cohort. The 4-year OS, PFS, LC, RRFS and DMFS rates were 75.7%, 64.0%, 87.2%, 80.1% and 66.7%, respectively. The OS rate in patients

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NPC cells typically express a limited set of EBV latent genes, including EBV nuclear antigen 1, latent membrane proteins (LMP1, LMP2A and LMP2B), and EBV-encoded small RNA7. LMP1 was first discovered in 198423 and has been shown to be one of the few essential genes for growth transformation of B lymphocytes in vitro. It is the only EBV-encoded protein shown to transform rodent fibroblasts24,25. It has been well established in laboratory studies that LMP1 participates in NPC pathogenesis and progression20,26. In this study, pre-treatment LMP1 was detected in nasopharyngeal swab specimens using PCR analysis. To our knowledge, our series represents the first attempt to evaluate the relationship between pre-treatment LMP1 status in nasopharyngeal swab specimens and long-term survival of loco-regionally advanced NPC patients. We report that the 4-year OS rate of the LMP1-negative group is significantly higher than that of LMP1-positive group. These results may be of clinical significance because it suggests that a relatively simple and non-invasive test can select patients with a potentially adverse outcome prior to any treatment. These high-risk patients may benefit from more intensive therapy, such as neoadjuvant chemotherapy.

A number of studies on the association between pre-treatment LMP1 expression and outcome have been reported. In a study with 44 patients accrued over two decades, Dietz et al.11 reported that histological type was the dominant factor affecting prognosis, with LMP1 expression on immunohistochemistry not a significant factor. Two studies from Turkey and North African, using immunohistochemistry in tumour biopsies, also failed to find clear correlation between the expression of LMP1 and NPC long-term outcome12,13. On the other hand, a study of 56 stage III–IV NPC patients by Haririwiyanto et al.15 demonstrated a significantly higher response rate and 2-year OS in patients without LMP1 expression at diagnosis compared with those with LMP1 expression. Similarly, Chen et al.14 reported that the 5-year OS for 224 patients with high or low expression of LMP1 was 54% and 68%, respectively (P = 0.020). Multivariate analysis showed that high expression of LMP1 was an independent adverse prognostic factor in these NPC patients. In contrast, Hu et al.16 found that LMP1-positive tumours grew faster than LMP1-negative tumours, but nevertheless had a better prognosis. On balance, a recent meta-analysis17 on 12 case-control studies comprising 718 cases has demonstrated, using the random effects model, that the probability of metastasis in NPC patients with LMP1 expression is 2.27 times higher than those without LMP1 expression. However, most of the prior studies used immunohistochemical methods to test LMP1 on biopsy tissue, which is invasive because tissue is required, and subjective, because different antibodies and counting methodologies may give varying results11,12.

In the present study, we found similar results using a molecular technique without the need for a biopsy. The 4-year OS in LMP1-negative group was significantly higher than that in the LMP1-positive group. LMP1 was the only independent prognostic factor for OS rate in our cohort. In addition, we showed clear trends towards

**Discussion**

Table 2: Four-year outcome by different latent membrane protein 1 (LMP1) status

<table>
<thead>
<tr>
<th>Rate/%</th>
<th>LMP-1 positive (N = 75)</th>
<th>LMP-1 negative (N = 12)</th>
<th>( \chi^2 )</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS</td>
<td>71.6</td>
<td>100.0</td>
<td>4.470</td>
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<tr>
<td>PFS</td>
<td>60.7</td>
<td>91.7</td>
<td>3.600</td>
<td>0.058</td>
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<tr>
<td>LC</td>
<td>84.9</td>
<td>100.0</td>
<td>1.925</td>
<td>0.165</td>
</tr>
<tr>
<td>RRFS</td>
<td>76.6</td>
<td>100.0</td>
<td>3.153</td>
<td>0.076</td>
</tr>
<tr>
<td>DMFS</td>
<td>62.0</td>
<td>91.7</td>
<td>3.363</td>
<td>0.067</td>
</tr>
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</table>

OS, overall survival; LC, local control; RRFS, regional relapse-free survival; DMFS, distant metastasis-free survival.

better RRFS, DMFS and PFS rates in the group without LMP1 expression. The discrepancy of results between different centres remains unexplained. However, the different sample sizes, regional distribution or different LMP1 variants, different technique of detection and different treatment techniques may all play a role. Compared with previous studies, our study demonstrated a more effective and non-invasive way of LMP1 assessment for NPC prognostic analysis.

The current study confirms that a non-invasive and easily administered test can offer a high detection yield when used with molecular detection methods. We detected LMP1 in 75 out of 87 samples. This yield is consistent to that demonstrated by other groups, such as Lin et al. Barring the possibility of sampling error in some patients, one possible reason for the absence of LMP1 in those 12 patients may be that LMP1 negativity may represent a secondary change within the tumour as a regulatory response to immunoselection. LMP1 is immunogenic and may evoke a host response. Previous authors have also demonstrated that LMP1 can induce rejection of transfected murine mammary carcinoma cells. These LMP1-negative tumours may have become independent of the proliferation-driving effect of LMP1 by secondary cellular changes, possibly by methylation of the putative control region of the LMP1 gene.

Some limitations of our study need to be addressed. All patients received neoadjuvant chemotherapy, but only 31 and 36 patients received concurrent chemotherapy and adjuvant chemotherapy, respectively, which is the current standard of care. However, patients were treated in a protocolized fashion, and any differences in outcome seen are unlikely to be treatment related. Because this procedure is non-invasive and is not optically guided by nasendoscopy, there is a risk of sampling error. However, in this and another study, this technique appears to be feasible with some operator training. Another shortcoming is that the molecular technique was not quantitative, and we were unable to measure the copy numbers of LMP1. However, with this technique of nasopharyngeal swab, which may extract variable quantities of epithelial carcinoma cells, the copy number may also be difficult to interpret.

The utilization of this and other known molecular prognostic factors, such as serum EBV DNA levels, or functional imaging techniques, may stratify the patient prior to any treatment into high- or low-risk groups allowing for individualized therapy.

![Figure 2: Kaplan–Meier curves by latent membrane protein 1 (LMP1) status: (A) overall survival; (B) progression-free survival; (C) local control; (D) regional relapse-free survival; (E) distant metastasis-free survival.](image)
One strategy could be to offer neoadjuvant chemotherapy to patients at higher risk of distant metastases. LMP1 itself may present as a therapeutic target, with a specific antibody against LMP1 showing some promise in the preclinical setting. As IL-21 against LMP1 showing some promise, LMP1 itself may present as a therapeutic option in this malignancy as well.

Conclusion

In conclusion, we present the first report that detection of LMP1 in nasopharyngeal swab specimens using PCR is associated with a poor prognosis. This adds to the literature on NPC prognostic factors and has a potential for development of future therapeutic options for this patient cohort.

Acknowledgements

QG designed the study, accrued patients and drafted the manuscript. IT interpreted the data and critically revised the manuscript. SL helped design the study and accrued patients. YS and ZC collected and analysed the data. JL, LH and QL accrued patients and assisted in analysis and interpretation. JP and JYL designed and conceptualized the study. All authors read and approved the final manuscript.

References