A lncRNA signature associated with tumor immune heterogeneity predicts distant metastasis in locoregionally advanced nasopharyngeal carcinoma

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Increasing evidence has revealed the roles of long noncoding RNAs (lncRNAs) as tumor biomarkers. Here, we introduce an immune-associated nine-lncRNA signature for predicting distant metastasis in locoregionally advanced nasopharyngeal carcinoma (LA-NPC). The nine lncRNAs are identified through microarray profiling, followed by RT-qPCR validation and selection using a machine learning method in the training cohort (n = 177). This nine-lncRNA signature classifies patients into high and low risk groups, which have significantly different distant metastasis-free survival. Validations in the Guangzhou internal (n = 177) and Guilin external (n = 150) cohorts yield similar results, confirming that the signature is an independent risk factor for distant metastasis and outperforms anatomy-based metrics in identifying patients with high metastatic risk. Integrative analyses show that this nine-lncRNA signature correlates with immune activity and lymphocyte infiltration, which is validated by digital pathology. Our results suggest that the immune-associated nine-lncRNA signature can serve as a promising biomarker for metastasis prediction in LA-NPC.
A pproximately 70% of patients with nasopharyngeal carcinoma (NPC) present with locoregionally advanced disease. In recent years, significant improvements in the local control of locoregionally advanced NPC (LA-NPC) have been made with the implication of intensity-modulated radiotherapy (IMRT). However, 20% of patients develop distant metastasis after radical treatment, making it the major contributor to NPC-associated deaths. Currently, the most widely used prognostic indicators for NPC are the tumor-node-metastasis (TNM) stage and circulating Epstein-Barr virus (EBV) DNA load. The TNM stage is an anatomically based system, and circulating EBV DNA consists of short DNA fragments released by NPC cells, the level of which is highly correlated with tumor size. However, it has been reported that patients with the same stage and similar EBV DNA levels experience a variety of clinical outcomes, indicating the insufficiency of these anatomical prognostic factors. Moreover, NPC is characterized by the heterogeneous nature and complex regulatory network within the tumor. Therefore, molecular biomarkers may provide more insightful information for NPC prognosis.

The discovery and characterization of long noncoding RNAs (lncRNAs) have gained widespread attention because of their various regulatory functions in biological processes. It is generally recognized that lncRNAs are a group of transcripts that are exquisitely regulated and are more cell-type-specific to a greater degree than mRNAs. Accumulating evidence correlates lncRNA dysregulation to human diseases, including cancers. To date, a large number of lncRNAs have been reported to facilitate tumor growth, migration and invasion, and to modulate immune response and signaling pathways, thus contributing to distant metastasis. Consequently, prognostic lncRNA signatures have been developed, and have shown promising accuracy in predicting tumor metastasis. However, few studies have yet reported whether lncRNA signatures could serve as metastasis predictors for patients with NPC.

A growing number of studies have confirmed the role of lncRNAs as prognostic biomarkers in multiple cancers, primarily based on RNA-seq and microarray data from public databases. However, the biomarkers developed by high-throughput methods lack the generalizability required for clinical translation due to the high cost of RNA-seq and microarray techniques. Quantitative reverse transcription-polymerase chain reaction (RT-qPCR), a universal and cost-effective approach, may make biomarkers feasible for use in most hospitals. However, caution should be taken when using different methods such as RNA-seq and RT-qPCR to generate different formulas and cutoff values due to their technical heterogeneity. Some methods are often difficult to translate to the real world, arguably due to the inaccessibility or complexity of the calculation formula and lack of independent cohort validation. Hence, an easily measurable prognostic lncRNA biomarker that stands up to validation is highly desirable.

In this multicenter cohort study, we introduce a nine-lncRNA signature as a robust predictor of LA-NPC metastasis. Global lncRNA expression is initially profiled with microarrays in the discovery cohort, which identifies metastasis-related lncRNAs. By applying RT-qPCR assay, we detect metastasis-related lncRNAs in 177 tissue samples from the training cohort and then select nine lncRNAs to construct a signature using a machine learning method. The signature can distinguish patients with increased metastatic risk and provide more accurate prediction than traditional clinical factors, which is validated by two independent cohorts. Bioinformatic analysis is used to explore the immune-associated characteristics of the nine-lncRNA signature and then, digital pathology is applied to confirm that the signature reflects the immunological heterogeneity within the tumor micro-environment (TME) of NPC. In summary, we identify and validate an immune-associated lncRNA signature that can serve as a promising tool for metastasis prediction in LA-NPC.

Results

Patient characteristics. We included 542 nonmetastatic LA-NPC patients and 18 healthy controls in this study (Fig. 1). In the discovery cohort, 18 LA-NPC patients and 18 healthy controls were matched by age and sex (Supplementary Table 1), and ten pairs of LA-NPC patients developed with or without posttreatment distant metastasis were matched based on additional tumor characteristics (T and N stage) and treatment modalities (Supplementary Table 2). The patient characteristics of the Guangzhou training cohort (n = 177), Guangzhou internal validation cohort (n = 177), and Guilin external validation cohort (n = 150) are shown in Table 1. The median follow-up was 83.5 months (interquartile range (IQR) 61.7–94.9), 82.1 months (IQR 60.3–96.4), and 49.4 months (IQR 39.9–62.0) in the training, internal and external cohorts, respectively.

Development of a nine-lncRNA signature for NPC metastasis. Based on the lncRNA microarray data, 1453 lncRNAs were differentially expressed between the tumor and normal tissues, and 525 lncRNAs were differentially expressed between the matched metastatic and nonmetastatic LA-NPC tissues. Overall, there were 149 lncRNAs at the intersection of these two comparisons (Supplementary Data 1), displaying strong classification properties (Supplementary Fig. 1a, b).

To construct a simple and practical prognostic model, we detected the expression of the 149 lncRNAs by RT-qPCR assay and then used a machine learning method to reduce the number of candidates. Twenty-eight lncRNAs were excluded from further study, as their expression was below detection in more than half of the samples. After univariate Cox analysis, we adopted the least absolute shrinkage and selection operator (LASSO) method to construct a lncRNA signature for predicting NPC metastasis in the training cohort. We generated a risk score using a formula that included the nine selected lncRNAs weighted by their regression coefficients in the penalized Cox model as follows: Risk score = (0.117 × expression of lnc-TRAPPC6B-2) + (0.042 × expression of lnc-DRD5-10) + (–0.054 × expression of NR2F2-AS1) + (0.077 × expression of lnc-CETP-1) + (0.121 × expression of lnc-CDK1-1) + (0.100 × expression of LINCO2065) + (0.196 × expression of lnc-POTEH-7) + (–0.409 × expression of lnc-STX6-2) + (–0.109 × expression of lnc-C11orf91-2). Then, an optimal cutoff value (1.007, AUC = 0.78) was selected via receiver operating characteristic (ROC) curve analysis.

Performance of the nine-lncRNA signature for NPC metastasis. With the optimal cutoff value, 51 patients in the training cohort were classified into the high-risk group, and the remaining 126 patients were classified into the low-risk group. Patients in the high-risk group had poorer distant metastasis-free survival (DMFS; HR 6.04, 95% CI 2.82–12.94, P = 1.4e–07), disease-free survival (DFS; HR 2.34, 95% CI 1.33–4.10, P = 2.2e–03) and overall survival (OS; HR 3.79, 95% CI 1.95–7.35, P = 2.3e–05) than those in the low-risk group (Fig. 2a–c). The number of patients who had an event for each risk group is listed in Supplementary Table 3.

Univariate analysis showed that the lncRNA signature was significantly associated with DMFS (Fig. 3). Other prognostic factors of DMFS included N stage and EBV DNA (N stage: HR 3.75, 95% CI 1.66–8.46, P = 0.002; EBV DNA: HR 3.61, 95% CI 1.48–8.84, P = 0.005; Fig. 3). Multivariate analysis revealed that...
the nine-lncRNA signature, N stage and EBV DNA remained independent prognostic factors for DMFS (the nine-lncRNA signature: HR 5.10, 95% CI 2.36–11.01, P = 3.5e-05; N stage: HR 2.41, 95% CI 1.04–5.56, P = 0.039; EBV DNA: HR 2.86, 95% CI 1.15–7.08, P = 0.023; Supplementary Table 4).

Validation of the nine-lncRNA signature in two independent cohorts. To further confirm the value of the nine-lncRNA signature in identifying metastasis, we validated our findings in the other two independent cohorts, using the same formula and cutoff developed in the training cohort. We categorized 137 of 177 patients from the Guangzhou internal validation cohort into the low-risk group and 40 patients into the high-risk group, and the two groups were found to have significant differences in terms of DMFS, DFS, and OS (DMFS: HR 6.63, 95% CI 3.09–14.20, P = 2.1e-08; DFS: HR 4.05, 95% CI 2.23–7.34, P = 6.1e-07; OS: HR 5.39, 95% CI 2.71–10.73, P = 7.8e-08; Fig. 2d–i). Then, 32 out of 150 patients from the Guilin external validation cohort were classified into the high-risk group, which were found to have shorter DMFS, DFS, and OS (DMFS: HR 5.97, 95% CI 2.90–12.28, P = 3.6e-08; DFS: HR 2.74, 95% CI 1.58–4.76, P = 1.9e-04; OS: HR 3.87, 95% CI 1.90–7.87, P = 5.6e-05; Fig. 2g–i). The nine-lncRNA signature was subsequently confirmed as a prognostic factor for DMFS in the internal and external validation cohorts (Fig. 3). After multivariable adjustment, the nine-lncRNA signature remained a powerful independent indicator of DMFS (Guangzhou internal cohort: HR 6.71, 95% CI 3.12–14.43, P = 1.1e-06; Guilin external cohort: HR 6.38, 95% CI 3.09–13.18, P = 5.3e-07; Supplementary Tables 5, 6).

The nine-lncRNA signature outperforms traditional clinical factors. We then compared the performance of the nine-lncRNA signature with that of N stage and EBV DNA for the prediction of metastasis, as these factors were significantly associated with DMFS in univariate Cox analysis. The results of the ROC analysis showed that the nine-lncRNA signature demonstrated superior efficacy than the N stage or EBV DNA alone in the training and two independent validation cohorts (Fig. 4a–c).

In addition, the nine-lncRNA signature and N stage were independent predictors of NPC metastasis in multivariate analyses after adjustment by other clinical variables (Supplementary Tables 4–6). To develop a more accurate prognostic tool, we built a model by combining the nine-lncRNA signature and N stage in the training cohort (Supplementary Table 7). When compared with the N stage alone, the combined model significantly improved the efficiency in predicting metastasis in the training cohort (AUC$_N$ stage = 0.65; AUC$_{combined}$ = 0.77; P = 0.005, Fig. 4d), which was corroborated in two validation cohorts (Guangzhou internal validation cohort: AUC$_{N}$ stage = 0.63; AUC$_{combined}$ = 0.80; P = 0.003; Guilin external validation cohort: AUC$_{N}$ stage = 0.62; AUC$_{combined}$ = 0.76; P = 0.007; Fig. 4e, f).

Biological characteristics related to the nine-lncRNA signature. To explore the function of the nine lncRNAs in the signature, we performed functional enrichment analysis with the microarray data of LA-NPC samples from the discovery cohort. In addition to the pathways highly correlated with tumorigenesis and metastasis, such as Myc targets and epithelial-mesenchymal transition (EMT), many of the nine lncRNAs were also associated with immune-related pathways (Fig. 5a). To investigate this finding more deeply, we focused on 17 immunologically relevant gene sets derived from the ImmPort website.$^{27}$ The results further demonstrated that the nine lncRNAs were involved in immune pathways, especially those related to inflammatory cytokines, T cell receptor (TCR) signaling, and B cell receptor (BCR) signaling (Fig. 5b).

Next, we explored whether the nine-lncRNA signature correlated with immune function, similar to the nine individual
In lncRNAs. By comparing gene expression in the high-risk and low-risk groups divided based on the lncRNA signature, we discovered that while genes upregulated in the high-risk group were primarily enriched in malignant property-related and metabolism-related pathways, genes upregulated in the low-risk group were mainly enriched in immune-related pathways, suggesting that the lncRNA signature may distinguish tumors between patients with high- and low-risk of distant metastasis. Furthermore, the lncRNA signature outperformed N stage and EBV DNA and provided additional prognostic value to these existing predictors. Our findings demonstrate the utility of lncRNAs as a metastasis prediction tool in LA-NPC patients. In this multicenter, retrospective cohort study, we identified an immune-associated signature in LA-NPC patients based on nine lncRNAs. The lncRNA signature improved the prognostic stratification of patients with LA-NPC by effectively distinguishing between patients with high- and low-risk of distant metastasis. Furthermore, the lncRNA signature outperformed N stage and EBV DNA and provided additional prognostic value to these existing predictors. Our findings demonstrate the utility of lncRNAs as a metastasis prediction tool in LA-NPC patients.

Association of the nine-lncRNA signature and lymphocyte infiltration. As the genes representing different immune cell types were differentially expressed in the high- and low-risk group, we investigated whether the immune infiltration pattern also differed between the two groups. Immune infiltration was estimated by the MCP-counter algorithm with microarray data. By performing RT-qPCR assay, we calculated the risk score for LA-NPC patients in the discovery cohort to distinguish between high- and low-risk patients. Patients in the low-risk group had a greater level of B cell and CD8⁺ T cell infiltration (Fig. 6a). To validate the result of the bioinformatic analysis, we performed hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) analyses. To prevent subjective interpretation of the pathologic results, we adopted a digital pathology method (Fig. 6b). The results revealed different immune infiltration in NPC tissues (Fig. 6c) and confirmed that B cells and intratumor CD8⁺ T cells were significantly more abundant in low-risk patients (Intratumoral CD20⁺ B cells, P = 0.011; striomal CD20⁺ B cells, P = 0.031; intratumoral CD8⁺ T cells, P = 0.045; Fig. 6d). Striomal CD8⁺ T cells showed a similar tendency but with marginal significance (striomal CD8⁺ T cells, P = 0.058; Fig. 6d).

Discussion
In this multicenter, retrospective cohort study, we identified an immune-associated signature in LA-NPC patients based on nine lncRNAs. The lncRNA signature improved the prognostic stratification of patients with LA-NPC by effectively distinguishing between patients with high- and low-risk of distant metastasis. Furthermore, the lncRNA signature outperformed N stage and EBV DNA and provided additional prognostic value to these existing predictors. Our findings demonstrate the utility of lncRNAs as a metastasis prediction tool in LA-NPC patients. Distinct metastasis is the primary reason for treatment failure in patients with NPC⁴, and clinicopathologic factors show relatively poor performance in distinguishing patients at high risk⁵. Luckily, recent studies have revealed the diverse functions of lncRNAs in regulating cancer metastasis, suggesting that lncRNAs could serve

| No. of patients | Low risk no. (%) | High risk no. (%) | P  |
|----------------|-----------------|------------------|----|
| Age            |                 |                  |    |
| <45 years      | 102 (60.3)      | 26 (51.0)        | 0.332 |
| ≥45 years      | 75 (39.7)       | 25 (49.0)        | 0.948 |
| Sex            |                 |                  |    |
| Male           | 140 (99.7)      | 41 (80.4)        | 0.950 |
| Female         | 37 (27.4)       | 10 (19.6)        | 0.917 |
| T stage        |                 |                  |    |
| T1             | 5 (3.2)         | 1 (2.0)          | 0.723 |
| T2             | 10 (7.9)        | 6 (11.8)         | 0.086 |
| T3             | 116 (85.7)      | 30 (57.5)        | 0.014 |
| T4             | 40 (27.1)       | 13 (25.5)        | 0.986 |
| N stage        |                 |                  |    |
| N0             | 16 (10.3)       | 3 (5.9)          | 0.086 |
| N1             | 76 (40.7)       | 16 (31.4)        | 0.037 |
| N2             | 51 (32.6)       | 18 (35.3)        | 0.973 |
| N3             | 34 (20.5)       | 14 (27.5)        | 0.450 |
| EBV DNA (copies/ml) | 59 (46.8) | 20 (39.2) | 0.450 |
| <2000          | 79 (59.4)       | 31 (60.8)        | 0.450 |
| ≥2000          | 98 (67.3)       | 83 (60.6)        | 0.450 |
| Distant metastasis | 0.16-06   | 0.55-07          | 0.58-06 |
| Yes            | 10 (7.9)        | 20 (39.2)        | 0.005 |
| No             | 116 (92.1)      | 31 (60.8)        | 0.005 |
| Disease progression | 0.89-03   | 1.10-05          | 0.92-03 |
| Yes            | 28 (22.2)       | 22 (43.1)        | 0.005 |
| No             | 127 (77.8)      | 29 (56.9)        | 0.005 |
| Death          |                 |                  |    |
| Yes            | 16 (12.7)       | 20 (39.2)        | 0.005 |
| No             | 110 (87.3)      | 31 (60.8)        | 0.005 |
| The P values were determined using the two-tailed χ² test. Source data are provided as a Source Data file. TNM tumor-node-metastasis, EBV Epstein-Barr virus.
Regression analyses were used to estimate the hazard ratios. HR hazard ratio, CI confidence interval. Source data are provided as a Source Data file.

Fig. 2 Kaplan–Meier estimates survival curves for the high-risk and low-risk groups according to the nine-lncRNA signature. a–c Distant metastasis-free survival (a), disease-free survival (b), and overall survival (c) in the training cohort (n = 177). d–f Distant metastasis-free survival (d), disease-free survival (e), and overall survival (f) in the Guangzhou internal validation cohort (n = 177). g–i Distant metastasis-free survival (g), disease-free survival (h), and overall survival (i) in the Guilin external validation cohort (n = 150). The log-rank test was used to calculate P values (two-sided), and univariate Cox regression analyses were used to estimate the hazard ratios. HR hazard ratio, CI confidence interval. Source data are provided as a Source Data file.

as potential prognostic biomarkers for tumor patients. However, many lncRNA biomarker studies have been conducted based on data from the public databases, and have not been subjected to large-scale, independent validation. In the current study, we adopted a three-step strategy to develop and validate an lncRNA signature. Microarrays were used to detect global lncRNA expression in LA-NPC patients and metastasis-related lncRNAs were discovered in the discovery stage. In the training stage, we used RT-qPCR to quantify the selected lncRNAs in a larger cohort, and developed a nine-lncRNA signature. Finally, we confirmed the performance of the nine-lncRNA signature in two independent validation cohorts. We believe that the proposed lncRNA signature could be a promising predictive tool for metastasis of NPC.

LncRNAs play significant roles in gene regulation and have emerged as critical regulators of cancer metastasis. The functions of the nine lncRNAs in our signature are mostly unknown, except for that of NR2F2-AS1 and Inc-POTEH-7,
which have been reported to promote tumor invasion in multiple cancers. Therefore, we explored the function of these lncRNAs with functional enrichment analysis. Interestingly, the results showed that these lncRNAs are probably related to immune function. Recent studies have discovered that lncRNAs are crucial regulators of the differentiation and function of immune cells that significantly affect the TME and ultimately contribute to tumor metastasis. In light of this finding, we further analyzed differences in the level of immune infiltration in the high- and low-risk groups as defined by the lncRNA signature, and the results suggested that high-risk patients had deficient CD8⁺ T cell and B cell infiltration. In other words, this lncRNA signature is likely indicative of immune infiltration. Inspired by this result, we further collected available paraffin-embedded samples in Guangzhou cohorts and detected CD8⁺ T cell and B cell infiltration, and the analysis yielded similar results. These findings indicated that our lncRNA signature could effectively classify LA-NPC patients into high- and low-risk groups, perhaps based on the immune association of these lncRNAs.

This immune-associated lncRNA signature displayed better performance than the N stage with regard to predicting distant metastasis in LA-NPC. The reason might be that while the N stage reflects anatomical differences, the lncRNAs reflect the biological heterogeneity and immune diversity of tumors, providing deeper insights into a patient’s systemic status. Next, we proposed that the addition of the immune-associated lncRNA signature to the N stage may improve the prediction accuracy. Indeed, the combination of N stage and the immune-associated lncRNA signature further boosted the predictive accuracy. Thus, our signature provides a simple and effective method for improved patient stratification.

Our present study has several strengths. First, we developed an immune-associated lncRNA signature based on RT–qPCR data.

| Events (n) / patients (N) with risk factor | Events (n) / patients (N) without risk factor | HR(95%CI) | P value |
|------------------------------------------|---------------------------------------------|----------|--------|
| Age (≥ 45 years vs.<45 years)            |                                             |          |        |
| Guangzhou training cohort                | 17/75                                       | 13/102   | 1.94 (0.94–4.00) | 0.072 |
| Guangzhou internal cohort                | 13/83                                       | 15/94    | 1.02 (0.49–2.15) | 0.951 |
| Guilin external cohort                   | 21/100                                      | 9/50     | 1.18 (0.54–2.57) | 0.686 |
| Overall                                  | 51/258                                      | 37/246   | 1.42 (0.93–2.17) | 0.106 |
| Sex (male vs. female)                    |                                             |          |        |
| Guangzhou training cohort                | 24/140                                      | 6/37     | 1.10 (0.45–2.68) | 0.842 |
| Guangzhou internal cohort                | 24/126                                      | 4/51     | 2.58 (0.89–7.43) | 0.079 |
| Guilin external cohort                   | 21/102                                      | 9/48     | 1.10 (0.50–2.40) | 0.812 |
| Overall                                  | 69/368                                      | 19/136   | 1.37 (0.83–2.28) | 0.220 |
| T stage (T3–4 vs. T1–2)                 |                                             |          |        |
| Guangzhou training cohort                | 24/156                                      | 6/21     | 0.53 (0.22–1.30) | 0.164 |
| Guangzhou internal cohort                | 25/159                                      | 3/18     | 0.94 (0.28–3.11) | 0.919 |
| Guilin external cohort                   | 23/122                                      | 7/28     | 0.74 (0.32–1.72) | 0.482 |
| Overall                                  | 72/437                                      | 16/67    | 0.66 (0.39–1.14) | 0.139 |
| N stage (N2–3 vs. N0–1)                 |                                             |          |        |
| Guangzhou training cohort                | 22/85                                       | 8/92     | 3.75 (1.66–8.46) | 0.002 |
| Guangzhou internal cohort                | 20/88                                       | 8/89     | 2.84 (1.25–6.44) | 0.013 |
| Guilin external cohort                   | 23/86                                       | 7/64     | 2.58 (1.11–6.02) | 0.028 |
| Overall                                  | 65/259                                      | 23/245   | 3.09 (1.92–4.98) | 3.6e-06 |
| EBV DNA (≥ 2000 vs.<2000 copies/ml)     |                                             |          |        |
| Guangzhou training cohort                | 24/98                                       | 6/79     | 3.61 (1.48–8.84) | 0.005 |
| Guangzhou internal cohort                | 22/108                                      | 6/69     | 2.53 (1.03–6.25) | 0.044 |
| Overall                                  | 46/206                                      | 12/148   | 3.03 (1.61–5.72) | 0.001 |
| LncRNA signature (high risk vs. low risk)|                                             |          |        |
| Guangzhou training cohort                | 20/51                                       | 10/126   | 6.04 (2.82–12.94) | 3.6e-06 |
| Guangzhou internal cohort                | 17/140                                      | 11/137   | 6.63 (3.09–14.20)| 1.2e-06 |
| Guilin external cohort                   | 16/32                                       | 14/118   | 5.97 (2.90–12.28)| 1.2e-06 |
| Overall                                  | 53/123                                      | 35/381   | 5.83 (3.80–8.94) | 7.0e-16 |

Fig. 3 Forest plots of the nine-lncRNA signature and clinicopathological characteristics on distant metastasis-free survival. Hazard ratios (HR), 95% confidence interval (CI), and P values were calculated by an unadjusted Cox proportional-hazards model with a two-sided Wald test. Squares represent the hazard ratios with error bars corresponding to 95% CI bounds. EBV Epstein-Barr virus, HR hazard ratio, CI confidence interval. Source data are provided as a Source Data file.
that is feasible to use in most hospitals and thus has great practical value in clinical settings. In addition, we subjected our signature to multivariable validation, proving the effectiveness of this promising tool. Moreover, the potential immune association of these lncRNAs was suggested by bioinformatic analyses and validated by digital pathology, although the sample size was limited. Finally, we acknowledge that this study was limited by its retrospective nature. These findings require confirmation in large-scale prospective studies.

In conclusion, we developed an immune-associated lncRNA signature consisting of nine lncRNAs that performed much better than clinical indicators in predicting the distant metastasis of LA-NPC. The signature is easily formulated using expression data that is feasible to use in most hospitals and thus has great practical value in clinical settings. In addition, we subjected our signature to multivariable validation, proving the effectiveness of this promising tool. Moreover, the potential immune association of these lncRNAs was suggested by bioinformatic analyses and validated by digital pathology, although the sample size was limited. Finally, we acknowledge that this study was limited by its retrospective nature. These findings require confirmation in large-scale prospective studies.

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In conclusion, we developed an immune-associated lncRNA signature consisting of nine lncRNAs that performed much better than clinical indicators in predicting the distant metastasis of LA-NPC. The signature is easily formulated using expression data that is feasible to use in most hospitals and thus has great practical value in clinical settings. In addition, we subjected our signature to multivariable validation, proving the effectiveness of this promising tool. Moreover, the potential immune association of these lncRNAs was suggested by bioinformatic analyses and validated by digital pathology, although the sample size was limited. Finally, we acknowledge that this study was limited by its retrospective nature. These findings require confirmation in large-scale prospective studies.
Fig. 5 Molecular characteristics of high-risk and low-risk groups. a Bar plot showing the number of lncRNAs out of the nine lncRNAs in the signature that are enriched in that corresponding pathway by functional enrichment analyses based on GSEA. Permutation-based P value shows the statistical significance of the normalized enrichment score (NES). Adjustments for multiple comparisons were presented by false discovery rate (FDR). Significantly enriched pathways are defined as P < 0.05, FDR < 0.25, and absolute NES > 1. b Bubble plot of GSEA results of the nine lncRNAs with immunologically relevant pathways in LA-NPC patients of the discovery cohort (n = 38). The red color of the dots represents enrichment (P < 0.05, FDR < 0.25, absolute NES > 1), and the size of the dots represents absolute NES. Permutation-based P value shows the statistical significance of NES and adjustments for multiple comparisons were presented by FDR. c Circos plot showing the significantly enriched pathways between the high- and low-risk groups based on GSEA. The size of each sector represents the number of genes in the labeled pathway. The color of the outermost circle and inner bar plots represent the category of pathways. The size of the second outer circle represents the percentage of genes contributing to the enrichment score, and its color represents the magnitude of the statistical significance (shown as −log10(P value)). The color of the third circle indicates that the labeled pathway is upregulated in the high- or low-risk group. The size of the inner bar plot shows the absolute NES. Permutation-based P value shows the statistical significance of NES and adjustments for multiple comparisons were presented by FDR. d Heatmap of differentially expressed immune-related genes between the high- and low-risk groups (Student’s two-sided t-test, P value < 0.05). FDR false discovery rate, NES normalized enrichment score. Source data are provided as a Source Data file.
Fig. 6 Immune infiltration of high-risk and low-risk groups. a Immune infiltration of patients (high-risk, n = 13; low-risk, n = 25) estimated by the MCP-counter algorithm. The dots represent the mean scores, and the error bars represent the standard deviation. The comparison was based on Student’s two-sided t-test. b Digital pathology analysis pipeline. For each sample, hematoxylin and eosin (H&E) and immunohistochemistry (IHC) slides were scanned, registered, and aligned in HALO software. Manual annotation of tumor nests and stroma areas on the H&E image were automatically synchronized to the IHC image. Positively stained cells in the IHC images were identified by the Multiplex IHC algorithm. Quantification of positively stained cells and the areas of tumor nests and stroma were automatically generated by HALO software. The scale bar represents 25 μm. c Representative images of CD20 and CD8 expression in intratumoral and stromal areas of tumor sections detected by IHC analysis in the high-risk (n = 21) and low-risk groups (n = 78). The images show high (left panel) or low (right panel) CD20+ B and CD8+ T cell densities in intratumoral and stromal areas, respectively. The scale bar represents 50 μm. d Comparison of CD20- and CD8-positive cells in intratumoral and stromal areas in patients in the high-risk (n = 21) and low-risk groups (n = 78) stratified by the nine-lncRNA signature. In each box plot, the centerline represents the median, the bounds represent the first and third quartiles and whiskers extend from the hinge to the largest value no further than 1.5 * interquartile range (IQR) from the hinge. Each dot represents a data point for individual patients. The comparison was based on Student’s two-sided t-test. Source data are provided as a Source Data file.
26,109 protein-coding transcripts. After sample labeling and array hybridization, raw data were extracted using the Agilent Feature Extraction software (version 11.0.1). Quantitative PCR, quality control, and differential expression analyses were performed with GeneSpring GX v12.1 software (Agilent Technologies). After normalization, transcript-specific probes for lncRNA labeled as “present” in more than or equal to half of the samples were considered high-quality for the biomarker selection. Differential expression analyses were performed based on t-test between samples from 16 LA-NPC and 18 matched healthy control pairs as well as between ten paired samples from LA-NPC developed with or without posttreatment distant metastasis. The lncRNAs showing significant differential expression between the two groups were identified with cutoffs of \(P < 0.05\) and fold change \(>1.5\). For integration of lncRNA and mRNA analysis, we only included samples from LA-NPC and removed the batch effect using the combat algorithm in “sva” package in R software. Protein-coding probes with flags “present” or “marginal” in more than or equal to ten samples were incorporated into the analysis.

### SmartChip qPCR analysis

Total RNA was reverse-transcribed using the GoScript™ Reverse Transcription System (Promega Corporation, Madison, Wisconsin, USA). The complementary DNA was analyzed by qPCR using Invitrogen™ Platinum™ SYBR Green SuperMix-UDG reagents (Thermo Fisher) with the Wafergen SmartChip Real-time PCR system (Takara Bio USA Inc., San Jose, CA). This system is suitable for large-scale gene expression studies, which can process 5184 nanowell reactions per run. After the initial denaturation at 95°C for 5 min, 45 cycles of the following program were used for amplification: 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s. The 72°C melting curve analysis was automatically generated by Wafergen SmartChip qPCR analysis software (version 2.8.6.1). After that, raw data of qPCR were also obtained with Wafergen SmartChip qPCR analysis software, excluding several nanowells with multiple melting peaks or amplification efficiency less than 1.5. lncRNA expressions were measured using the AICt method with AICt as the internal control gene (lncRNA Cq value the intra AICt Cq value within the same sample). The PCR primers used in this study were listed in Supplementary Table 8.

### Construction of the nine-lncRNA signature

To construct a lncRNA signature, we first used univariate Cox analysis to select lncRNAs related to DMFS in the training cohort. LASSO was adopted to further reduce the number of candidates by the “glmnet” package in R software. In short, a \(\lambda\) value was chosen via min (minimum error) criteria under ten-fold cross-validation. Based on the \(\lambda\) value, we could select the lncRNAs whose beta coefficients were not zero to calculate a risk score for each patient. The risk score was generated using a formula derived from the expression levels of these lncRNAs weighted by their coefficients and then a ROC curve was used to determine the optimal cutoff values with the “pROC” package in R software. Patients were divided into low- and high-risk groups with the threshold that produced the maximum sum of sensitivity and specificity in the ROC curve.

### Functional enrichment analysis

We investigated the functional contexts of the nine lncRNAs in our signature based on the guilt-by-association principle. This approach is based on correlation analysis between matching lncRNA and mRNA expression in combination with enrichment strategies. It contains three steps: (i) for each lncRNA, an expression correlation matrix (Spearman’s rank correlation) was constructed by integrating lncRNA and matching mRNA expression data; (ii) the expression-correlation matrix was ranked by correlation coefficient in descending order; and (iii) the sorted expression-correlation matrix was used as input for GSEA (gene set enrichment analysis). We used the nine-lncRNA signature as an input for GSEA. The publicly available hallmark gene lists used in this study are available in the Molecular Signatures Database (MSigDB) database (http://www.gsea-msigdb.org/gsea/index.jsp), and the immune-related gene lists available on ImmPort website (https://www.immport.org/home). The microarray data used in this study have been deposited at Gene Expression Omnibus under accession code GSE180272. The remaining data are available within the Article, Supplementary Information, or Source Data file.

### Code availability

Essential scripts for model development and validation in multiple cohorts are available at the Github website (https://github.com/TL-L26/lncRNA_signature_for_NPC)25.

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### Data availability

The publicly available hallmarks gene lists used in this study are available in the Molecular Signatures Database (MSigDB) database (http://www.gsea-msigdb.org/gsea/index.jsp), and the immune-related gene lists available on ImmPort website (https://www.immport.org/home). The microarray data used in this study have been deposited at Gene Expression Omnibus under accession code GSE180272. The remaining data are available within the Article, Supplementary Information, or Source Data file. Source data are provided with this paper.

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Competing interests
The authors declare no competing interests.
Additional information

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