Original Article

Comprehensive analysis of immune related lncRNAs in the tumor microenvironment of stage II–III colorectal cancer

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Background: Long non-coding RNAs (lncRNAs) associated with immunological function have increasingly been found to act as effective prognostic biomarkers of the overall survival (OS) of colorectal cancer (CRC) patients. We sought to identify a signature of immune-related lncRNAs that offered value as a tool for the prospective prognostic evaluation of patients with stage II–III CRC.

Methods: The clinical and gene expression data of CRC patients in The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) databases was obtained and separated into a training cohort composed of 202 samples, a test cohort of 124 samples from the GSE72970 dataset, and a validation cohort of 91 samples from the GSE143985 dataset.

Results: We firstly evaluated intratumoral immune cell infiltration by conducting a Single-sample gene set enrichment analyses (ssGSEA) analysis to separate patient tumors into those with low immune cell infiltration and those with high immune cell infiltration. We then compared lncRNA and mRNA expression profiles between these two tumor types, leading us to focus on eight lncRNAs identified within the resultant mRNA-lncRNA co-expression network. Multivariate Cox regression models were then utilized to detect an immune-associated lncRNA signature that offered value for prognostic model construction. Functional analyses revealed this lncRNA signature to be associated with key immunological pathways including the JAK-STAT signaling, T cell receptor signaling, and Rap1 signaling pathways.

Conclusions: Together, our results suggest that our immune-related 4 lncRNA signature can reliably predict stage II–III CRC patient prognosis, thereby guiding efforts to better understand this disease and to effectively treat it.

Keywords: Colorectal cancer (CRC); overall survival (OS); prognosis; signature; long non-coding RNAs (lncRNAs)

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Introduction

Colorectal cancer (CRC) is the third most common form of cancer and the fourth leading cancer-related cause of mortality (1). While the use of immune checkpoint inhibitor (ICI) therapy has achieved success in improving the outcomes of CRC patients with mismatch repair-deficient and microsatellite instability-high (dMMR–MSI-H) tumors, the efficacy of this approach in other CRC subtypes has been less promising (2,3). Through multiple genetic and epigenetic alterations, tumors can inactivate tumor suppressor genes and induce oncogene activation, allowing cancer cells to evade detection by the immune

system and to metastasize by invading local and distal tissues (4,5). While the majority of CRC-related studies conducted to date have focused on patients with advanced disease, most CRC patients have stage II–III disease. To guide the most appropriate treatment of these patients, it is important that their tumor-associated immune responses be better understood, and through determining the regulatory mechanisms associated with overall survival (OS), it may be possible to better identify reliable therapeutic biomarkers or targets amenable to clinical targeting or evaluation.

Long non-coding RNAs (lncRNAs) over 200 nucleotides in length have been found to regulate proliferation, survival, chemoresistance, and metastasis in a range of cancers including CRC. Owing to their complex regulatory roles, several lncRNAs have been identified as potential diagnostic or prognostic biomarkers in various oncogenic contexts (5,6). For example, NEAT1 is a lncRNA associated with Wnt/β-catenin signaling that has been suggested to be a potential prognostic biomarker and/or target for therapeutic intervention in CRC (7), while BANCR is a novel CRC-related lncRNA that is important in the context of epithelial-mesenchymal transition (8). Similarly, the overexpression of HAGLR, GAS5, NEAT1, H19, PINT, and CRNDE, which are related to chromatin looping and the suppression of sense coding gene transcription, has been linked to a poor CRC prognosis (9). These prior results clearly demonstrate lncRNAs are associated with CRC onset and patient prognosis. The immune system is also a key regulator of cancer progression in affected patients (10,11), with immune-related genes commonly being dysregulated in many different cancers. For example, in CRC, the KRAS-IRF2 axis has been found to facilitate immunosuppression and resistance to therapeutic intervention, while elevated expression of ST2 has been linked to reduced CRC patient survival and impaired $CD8^+$ T cell cytotoxicity (12). By characterizing immune-related gene expression, it is thus possible to better understand the dynamics of intratumoral immune infiltration in CRC tumors.

The tumor microenvironment (TME) is composed of tumor cells, inflammatory cells, vasculature, tumor-associated fibroblast (CAF) and extracellular matrix (13), which can promote neoplastic transformation, increase tumor growth and invasion and avoid host immune function (14). TME is receiving more and more attention as a prognostic marker. Van den Eynde *et al.* quantified Immunoscore through calculated CD3 and CD8 densities from the tumor core (CT) and the invasive margin (IM) of metastases, revealed the heterogeneity of immune infiltrates for tumors (15). Then, Pagès *et al.* confirmed Immunoscore can reliably assess the risk of recurrence of CRC patients, based on an international prognostic and accuracy study of 14 centres in 13 countries (16). Therefore, finding the different omics prognostic biomarkers based on colorectal cancer tumor microenvironment will not only help to understand the development mechanism of colorectal cancer tumor microenvironment, but also help establish a new molecular subtype of colorectal cancer.

Single sample gene set Enrichment analysis (ssGSEA) is an extension of GSEA method, mainly designed for a single sample that cannot do GSEA. Rank normalization was performed for gene expression values of a given sample, and enrichment scores (ES) were calculated using empirical cumulative distribution functions. The scores of immune-related gene sets in each sample can obtained by ssGSEA (17). Herein, we employed ssGSEA analyses to differentiate between CRC tumors with low and high levels of immune infiltration and compared the expression of immune-related mRNAs and lncRNAs between these two tumor subtypes. This approach led us to identify eight key lncRNAs through the development of an mRNA-lncRNA co-expression network. Subsequently, multivariate Cox regression analyses were used to develop a 4-lncRNA expression signature that was associated with stage II–III CRC patient prognosis in a training cohort, a validation cohort, and in two independent cohorts. This immune-associated lncRNA signature offers value as a means of both predicting the prognosis of stage II–III CRC patients and of better understanding the progression and appropriate treatment of this deadly disease.

We present the following article in accordance with the STARD reporting checklist (available at https://dx.doi.org/10.21037/jgo-21-594).

Methods

Sample datasets

The Ensembl v69 assembly (http://www.ensembl.org) was utilized to generate FPKM (Fragments Per Kilobase of transcript per Million fragments mapped) values for downloaded gene expression datasets of interest, and clinical and gene expression data corresponding to 202 patients with stage II-III CRC were downloaded from The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/). Furthermore, gene expression and follow-up data

Table 1 Summary of patient demographics and clinical characteristics

Characteristics	TCGA	GSE72970	GSE143985
Samples	202	124	91
Gender, n (%)			
Female	109 (54.0)	50 (40.3)	61 (67.0)
Male	93 (46.0)	74 (59.7)	30 (33.0)
Age, n (%)			
<65 years	112 (55.4)	74 (59.7)	44 (48.4)
≥65 years	90 (44.6)	50 (40.3)	47 (51.6)
Stage, n (%)			
II	116 (57.4)	58 (46.8)	55 (60.4)
III	86 (42.6)	66 (53.2)	36 (39.6)
Vital status, n (%)			
Living	159 (78.7)	48 (38.7)	76 (83.5)
Dead	43 (21.3)	76 (61.3)	15 (16.5)

corresponding to other stage II–III CRC patients were downloaded from the Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo) datasets GSE72970 (n=124) and GSE143985 (n=91) (*Table 1*).

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Clustering

Single-sample gene set enrichment analyses (ssGSEA) scores were used to assess the enrichment of 29 different immune-related signatures in each individual sample (18), after which hierarchical clustering of these patient samples was conducted based upon enrichment scores.

Differentially expressed immune-related mRNA and lncRNA identification

The R limma package was utilized to identify lncRNAs and mRNAs that were differentially expressed when comparing CRC patient samples with low and high levels of immune cell infiltration, with significantly differentially expressed lncRNAs and mRNAs being those meeting the following criteria: P<0.05 and |log FC|>1. Immune-related lncRNA and mRNA were screened according to differences in immune infiltration.

Immune-related lncRNA-mRNA co-expression network construction

Pearson correlation coefficients corresponding to individual pairs of mRNAs and lncRNAs were calculated based upon their expression values. Those mRNA-lncRNA pairs meeting the criteria: |Pearson R| >0.8 and P<0.05, were then selected and used to generate a co-expression network using Cytoscape software, with the MCODE plug- in (v.3.4.2; http://www.cytoscape.org/) being employed to visualize this network.

Immune-related prognostic lncRNA signature construction

A multivariate Cox regression model was used to identify the immune-related lncRNAs offering the greatest prognostic utility in the training dataset as follows:

$$Risk\ Sore=\sum_{i=1}^{N} Exp_i * Coef_i \quad [1]$$

Where N corresponds to the number of prognostic lncRNAs, $Expi$ corresponds to the expression of these lncRNAs, and $Coefi$ is a corresponding single-factor Cox regression coefficient. The overall Risk Score (RS) was the multi-node weighted sum of individual risk scores.

Statistical analysis

R (v.3.6.0) was used for all statistical analyses. In the training dataset, median risk scores were utilized as cutoff values to separate stage II–III CRC patients into high- and low-risk cohorts (19). The OS of these two cohorts was then compared using Kaplan-Meier curves and two-sided log-rank tests. The prognostic utility of our immune-related lncRNA signature was estimated based upon receiver operatic characteristic (ROC) curves and Kaplan-Meier survival analyses. P<0.05 was the significance threshold for this study.

Construction of a lncRNA functional network

To understand the potential functional roles of immune-associated lncRNAs of interest, we conducted Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. Initially, the four lncRNAs correlated with differentially expressed mRNAs (|Pearson R| >0.8 and P<0.05), after which the mRNAs associated with these lncRNAs were subjected to functional analyses. Cytoscape

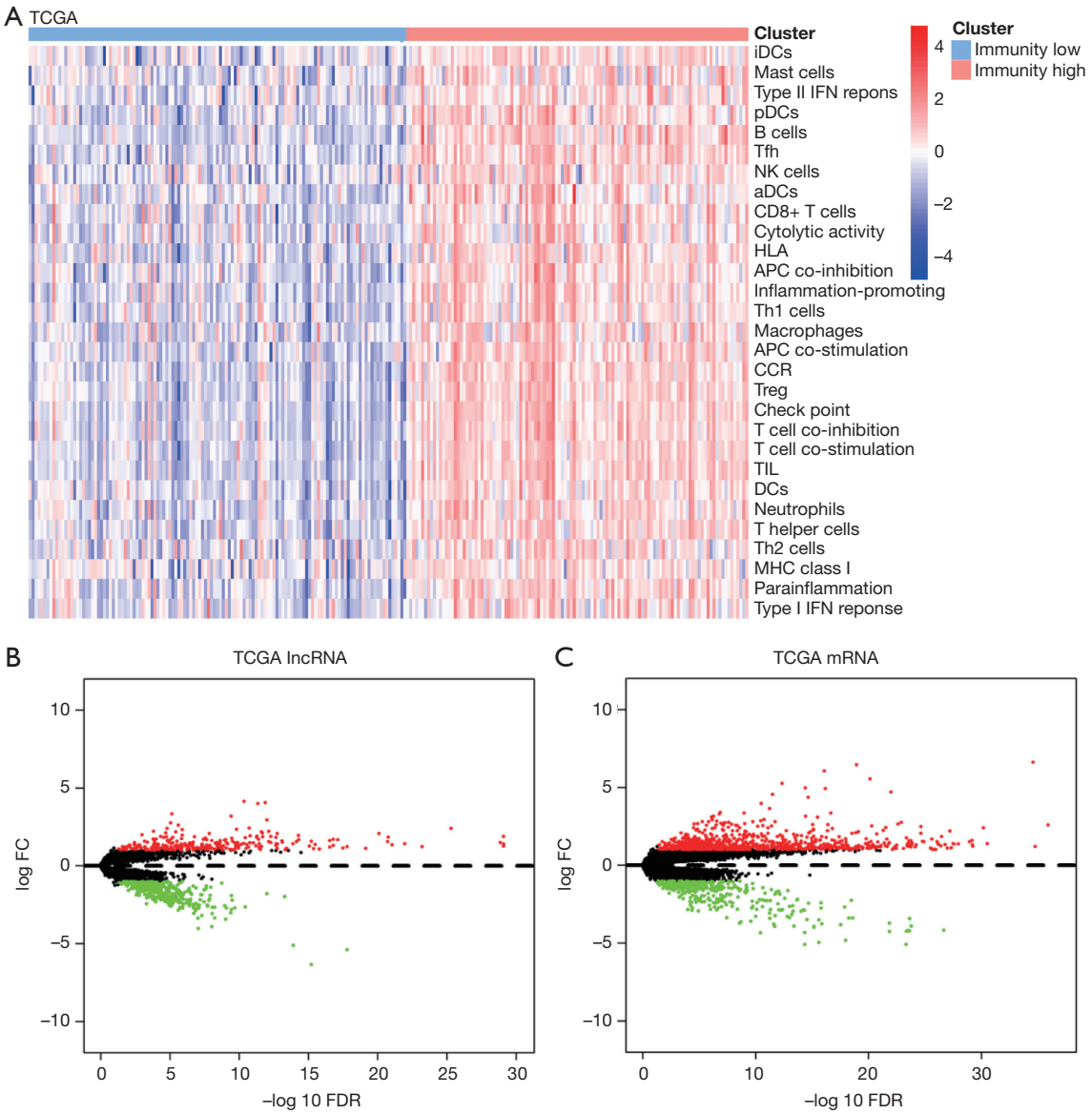

Figure 1 Expression profiles of stage II–III CRC subtypes in the TCGA cohort. Hierarchical clustering of stage II–III CRC yields high immune cell infiltration group and low immune cell infiltration subtypes (A). Differentially expressed mRNAs (B) and lncRNAs (C) between a high immune cell infiltration group and low immune cell infiltration subtypes in the TCGA cohort.

v.3.7.2 was then used to construct a lncRNA functional network based upon these database inputs and screening parameters.

Results

CRC sample immune subtype classification

We initially evaluated CRC patient samples in the TCGA dataset for the relative enrichment of 29 different immune-related gene sets associated with particular cell types and signaling pathways (20,21). Following ssGSEA score analyses of these 202 individual samples, we were able to hierarchically cluster them into samples with low and high levels of immune cell infiltration according to their relative enrichment for the different immune-related gene signatures (*Figure 1A*). Consistent with this approach, samples in the high immune infiltration sample subset had higher average immune scores than did those in the low immune infiltration sample subset.

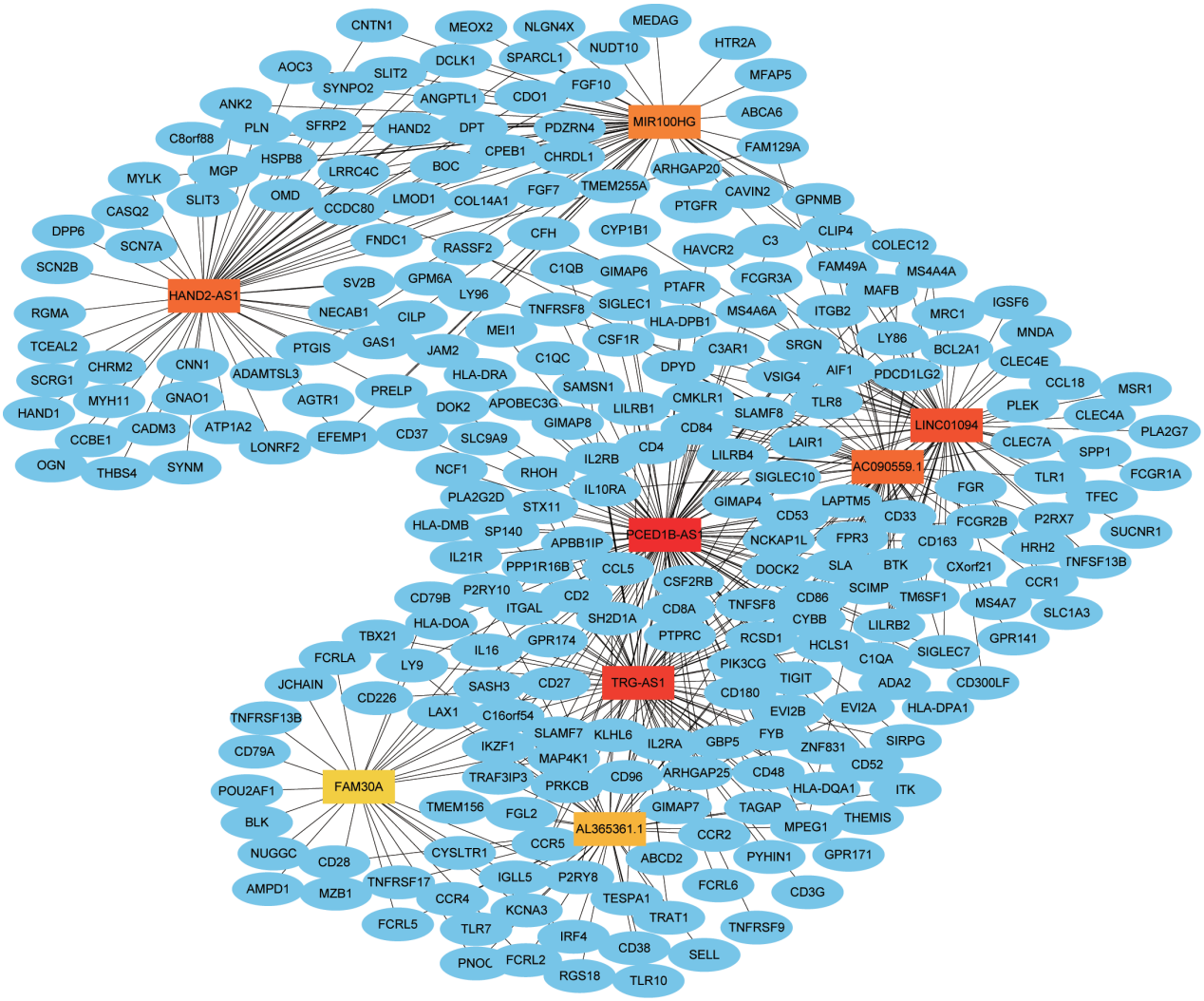

Figure 2 Immune-related lncRNAs-mRNAs co-expression analysis (|Pearson correlation coefficient| >0.4 and P value <0.05).

Immune-related mRNA and lncRNA co-expression network construction

We next compared gene expression data between TCGA samples in the low and high immune cell infiltration subsets, with the expression of 21,999 mRNAs and 6,829 lncRNAs being compared. This analysis enabled us to identify 1,152 differentially expressed mRNAs (353 downregulate, 799 upregulated) and 649 differentially expressed lncRNAs (385 downregulate, 264 upregulated) when comparing these two sample subsets using the cutoff criteria |Fold change| ≥2 and P<0.05 (*Figure 1B,1C*). To further understand the roles of these differentially expressed lncRNAs in stage II–III CRC and to understand their associations with key mRNAs of interest, we next generated a lncRNA-mRNA co-expression network that incorporated pairs of the differentially expressed mRNAs and lncRNAs that met the following screening criteria: |Pearson R| >0.8 and P<0.05. This analysis led to the recognition that several lncRNAs identified in our initial analysis (HAND2.AS1, MIR100HG, LINC01094, AC090559.1, FAM30A, AL265361.1, PCED1B.AS1, and TRG.AS1) were important regulators of stage II–III CRC (*Figure 2*).

Development of a prognostic immune-related lncRNA risk signature

As complete clinicopathological data were available for the 202 samples in the training dataset, we next evaluated the association between CRC patient OS and the expression

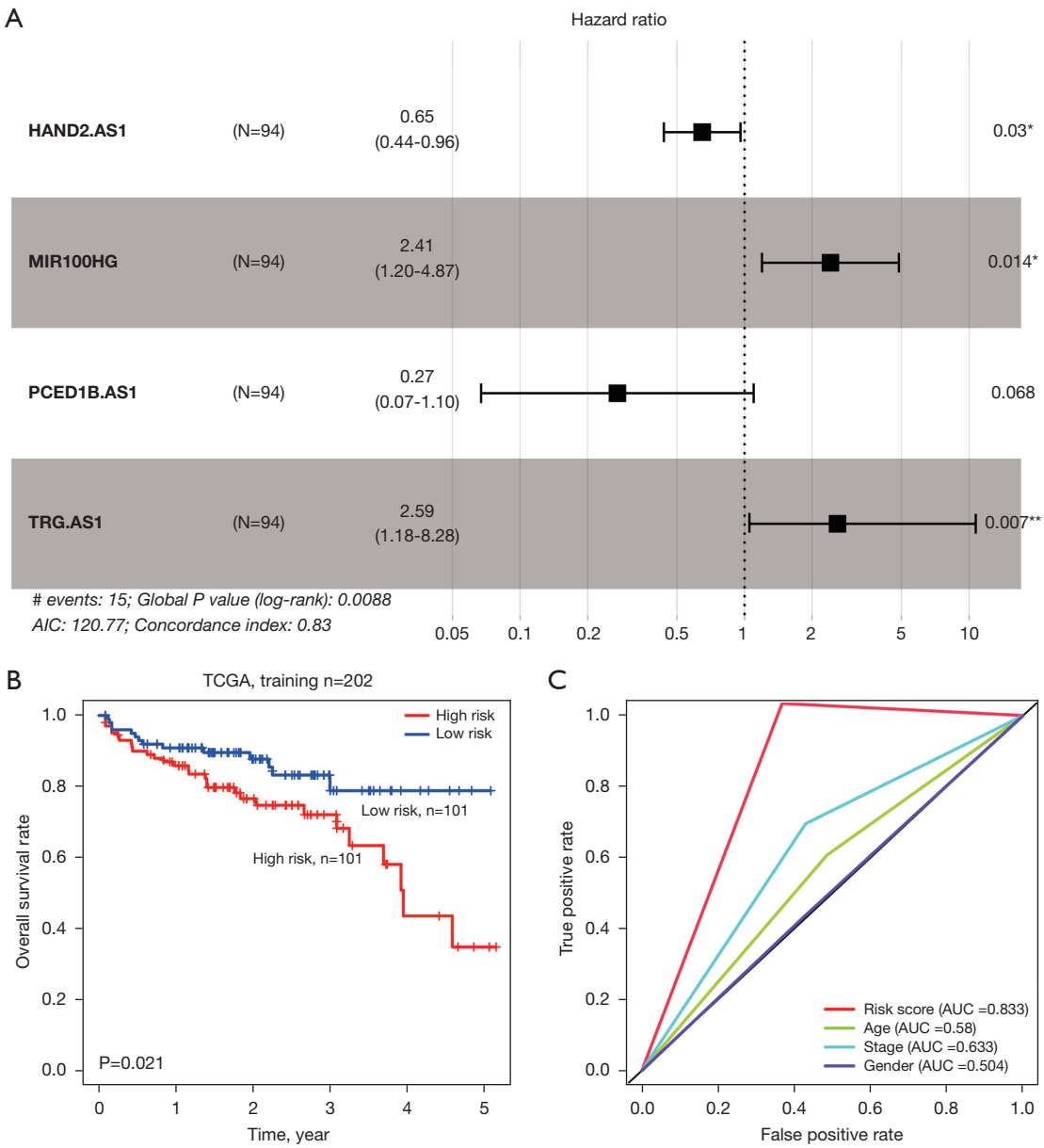

Figure 3 Construction of an immune-related lncRNA signature associated with prognosis using multivariate Cox regression analysis in the training dataset (A). Kaplan-Meier survival curves classified osteosarcoma patients into high-risk and low-risk groups using the lncRNAs signature in the training and test datasets. P values were calculated by log-rank test (B). Results of receiver operating characteristic (ROC) analysis (C). *, P<0.05, **, P<0.01, Log-Rank test.

of the eight core lncRNAs identified in the mRNA-lncRNA co-expression network. A multivariate Cox regression analysis (*Figure 3A*) was ultimately used to develop a 4-lncRNA risk signature model (HAND2.AS1, MIR100HG, PCED1B.AS1, and TRG.AS1) that could be used to estimate CRC patient survival. This model was used to generate risk scores (RS) (https://cdn.amegroups.cn/static/public/10.21037jgo-21-594-1.pdf) based upon the expression of the four different lncRNAs as follows:

$$RS = (-0.43 * Exp_{HAND2.AS1}) + (0.88 * Exp_{MIR100HG}) + (-1.3 * Exp_{PCED1B.AS1}) + (0.95 * Exp_{TRG.AS1}) \quad [2]$$

where *Exp* corresponds to lncRNA expression values.

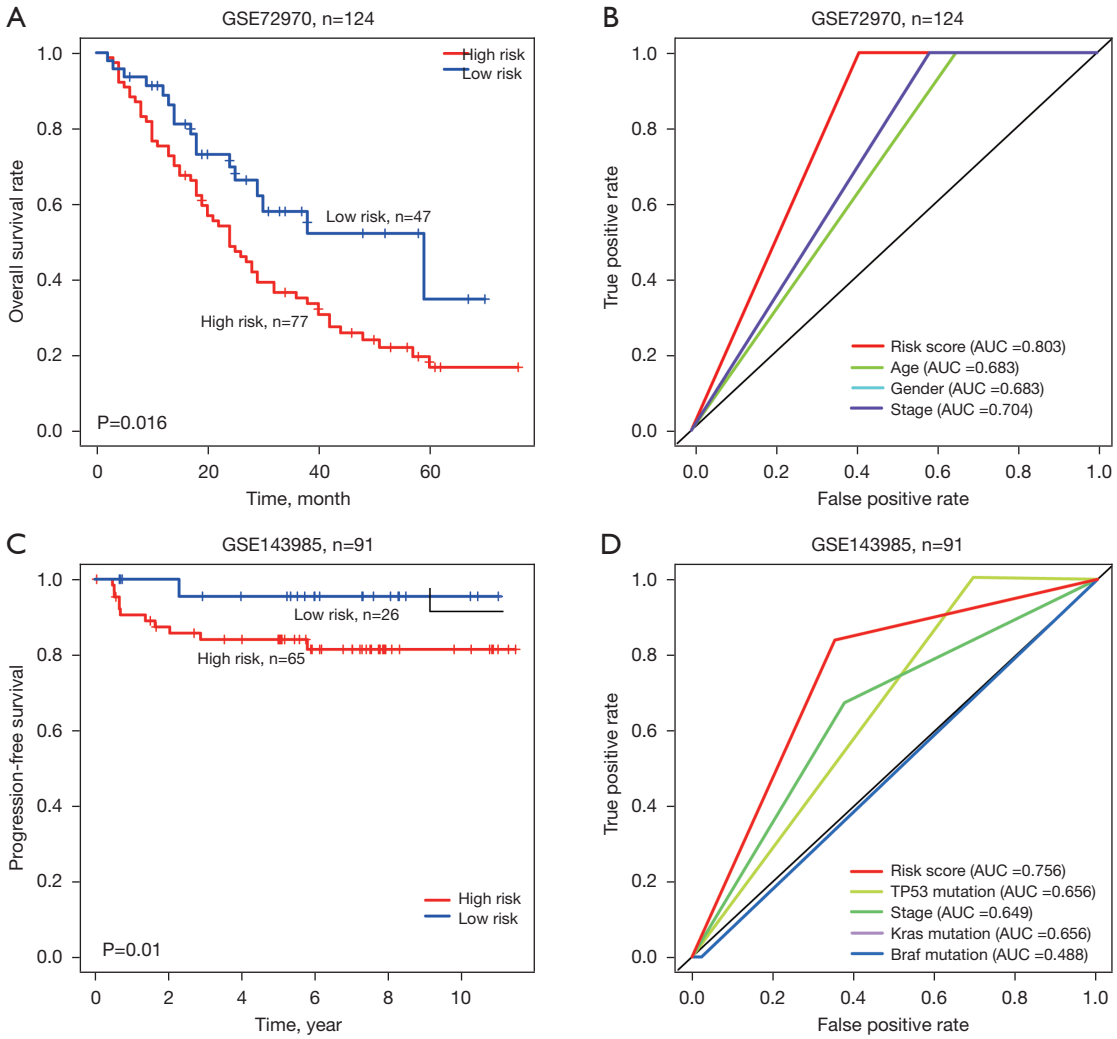

Figure 4 Kaplan-Meier survival and progression-free survival curves classified stage II–III CRC patients into high-risk and low-risk groups using the immune-related lncRNAs signature in the GSE72970 and GSE143985 datasets, respectively. P values were calculated by log-rank test (A,C). Results of receiver operating characteristic (ROC) analysis (B,D).

Evaluation of the prognostic relevance of this immune-related lncRNA risk signature in independent datasets

We next separated patients in the training cohort into high- and low-risk subsets based upon the median risk score generated using the 4-lncRNA risk signature model (n=101 each). The results showed patients in the high-risk group had a 5-year OS of <40%, whereas those in the low-risk group exhibited >80% 5-year OS [hazard ratio (HR): 2.72, 95% confidence interval (CI): 1.82–2.06, P=0.021] (*Figure 3B*). In the training dataset, the immune-related lncRNA signature was found to more reliably predict patient prognosis than any other clinical features ($AUC_{Signature}$ =0.833, AUC_{age} =0.58, AUC_{stage} =0.633, and AUC_{gender} =0.504) (*Figure 3C*).The prognostic utility of the model was then validated by using it to calculate immune-related lncRNA risk sores for the GSE72970 CRC dataset by separating high-risk and low-risk groups of the GSE72970 CRC dataset based on the median risk score calculated for training dataset. Survival outcomes between low- and high-risk samples in this dataset were compared via Kaplan-Meier curves (*Figure 4A*), and showed high-risk patients exhibited <20% 5-year OS, whereas low risk patients exhibited >30% 5-year OS (HR: 1.62, 95% CI: 1.32–3.76, P=0.016). This same approach was also used to calculate immune-related lncRNAs signature-based median risk

scores for the independent GSE143985 dataset, wherein high- and low-risk CRC patients exhibited significant differences in progression-free survival (HR: 2.14, 95% CI: 1.22–3.88, P=0.01).

Evaluation of the prognostic utility of the immune-related lncRNA signature

ROC curve analyses were employed to examine the relative predictive power of our immune-related lncRNA signature and of stage II–III CRC patient clinicopathological features, with a larger area under the ROC curve being consistent with better prognostic utility. Similarly to the training dataset, this lncRNA signature was superior to other clinical features in the GSE72970 dataset ($AUC_{Signature}$ =0.803, AUC_{age} =0.683, AUC_{stage} =0.706, and AUC_{gender} =0.683) (*Figure 4B*), this same approach was also used to calculate immune-related lncRNAs signature-based median risk scores for the independent GSE143985 dataset, wherein high- and low-risk CRC patients exhibited significant differences in progression-free survival (HR: 2.14, 95% CI: 1.22–3.88, P=0.01) (*Figure 4C*), and in the GSE143985 dataset ($AUC_{Signature}$ =0.756, $AUC_{TP53\_mutation}$ =0.58, $AUC_{KRAS\_mutation}$ =0.633, $AUC_{BRAF\_mutation}$ =0.488, and AUC_{stage} =0.649) (*Figure 4D*). These findings underscored the novelty and prognostic accuracy or our immune-associated lncRNA risk signature.

Functional analysis of the immune-related lncRNA risk signature

Lastly, we conducted KEGG analyses to understand the potential functional roles of the four lncRNAs in the development and progression of stage II–III CRC. To that end, mRNAs that were co-expressed with each of the lncRNAs (|Pearson R| >0.8 and P<0.05) were analyzed and the resultant functional network was visualized using Cytoscape (*Figure 5A*). This analysis revealed the four lncRNAs were associated with the ket immunological pathways including the JAK-STAT, T cell receptor, B cell receptor, and Rap1 signaling pathways. Specifically, we found that HAND2-AS1 and MIR100HG were associated with the Rap1 signaling pathway, cell adhesion molecules (CAMs), and the calcium signaling pathway, while PCED1B-AS1 and TRG-AS1 were associated with JAK-STAT signaling, Th17 cell differentiation, natural killer cell-mediated cytotoxicity, Th1 and Th2 cell differentiation, the chemokine signaling pathway, and T cell receptor signaling. Finally, we also validated the differences in immune-related lncRNA signature expression between low- and high-immune cell infiltration tumor subtypes in the GSE143985 dataset, yielding results similar to those from our TCGA cohort (*Figure 5B-5E*).

Discussion

CRC is one of the most studied forms of human cancer (22), yet the molecular basis for stage II–III CRC development and progression remains to be fully elucidated. Recent evidence has shown that lncRNAs play important regulatory roles in controlling intratumoral dynamics and the infiltration and activation of immune cells, with certain lncRNA signatures being characteristic of immune cell activation (23-25). For example, MALAT1 can sequester miR-195, thereby upregulating its target PD-L1 and promoting tumor immune evasion via altering $CD8^+$ T cell proliferation and survival (26). MIR155HG has also been found to be an immune checkpoint marker and potential prognostic biomarker associated with tumor immune cell infiltration (27). Furthermore, the high stromal expression of TBILA has been shown to promote TGF-b upregulation and EMT induction in non-small cell lung cancer (28). These prior findings underscore the relevance of immune-related lncRNAs to the prognosis of cancer patients. However, specific prognostic lncRNA signatures suitable for analyzing stage II–III CRC patient outcomes are lacking at present, and the present study was designed with the goal of identifying reliable biomarkers of OS outcomes in these patients.

Herein, we evaluated immune cell infiltration in CRC patient tumors in the TCGA dataset by conducting ssGSEA analyses, which allowed the separation of tumors based upon whether they exhibited low or high levels of immune infiltration. We then characterized lncRNAs and mRNAs that were differentially expressed between these two sample subsets, ultimately focusing on eight immune-associated lncRNAs that were found to be centrally located within an mRNA-lncRNA co-expression network. We further identified a 4-lncRNA risk signature that was associated with stage II–III CRC patient prognosis via multivariate Cox regression analyses, and validated its prognostic value in two independent CRC patient datasets.

Importantly, this lncRNA risk signature was found to offer greater prognostic value than any of the individual clinicopathological features of tumors in these patients. Functional analyses revealed the lncRNAs to be associated

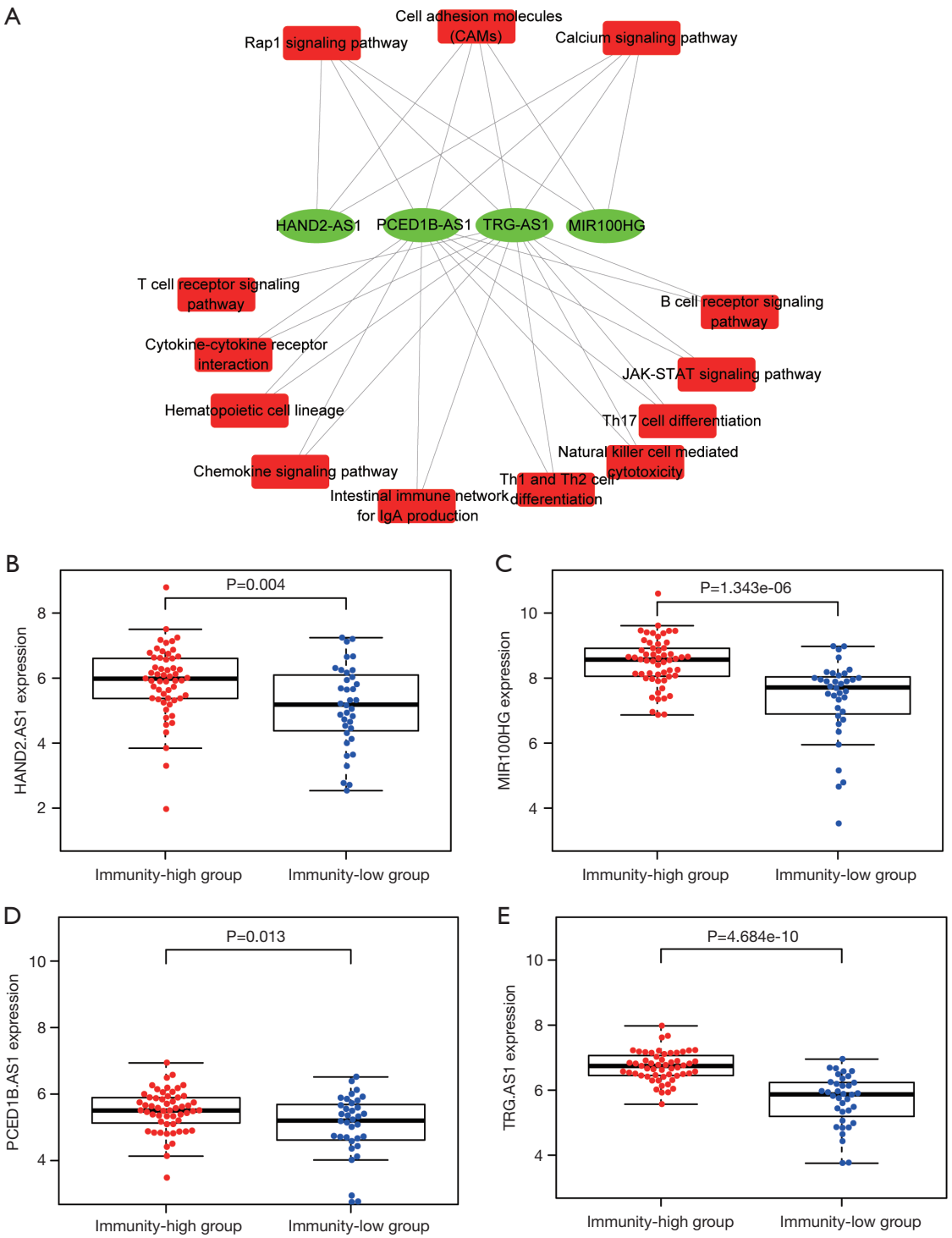

Figure 5 The four immune-related lncRNA-function network. Red rectangles represent functions and green diamonds (A). LncRNAs validated the expression of the immune-related lncRNA signature between high immune cell infiltration and low immune cell infiltration subtypes in the GSE143985 (B-E).

with key immune signaling pathways including the JAK-STAT, T cell receptor, B cell receptor, and Rap1 signaling pathways (1). We additionally validated the differential expression of the signature in patients with high and low levels of immune cell infiltration in the GSE143985 dataset, yielding findings comparable to the TCGA cohort analyses. Together, these findings indicate this immune-related lncRNA risk signature can reliably predict stage II–III CRC patient prognosis, while also offering novel insights into the mechanistic basis for the progression of this deadly disease.

At a functional level, we found HAND2.AS1 and MIR100HG to be associated with the Rap1 signaling pathway, CAMs, and the calcium signaling pathway. Rap1 is an important regulator of invasion and metastasis in many cancers (29), while CAMs are key regulators of the tumor microenvironment and associated immune responses (30). Prior analyses have also linked HAND2.AS1 and MIR100HG to tumor development, with the former having been shown to regulate NSCLC cell stemness, migration, and invasion via interacting with TGF-β1 (31). HAND2-AS1 has also been found to sequester miR-3118 and to inhibit SOCS5 signaling, thereby modulating JAK-STAT signaling and inhibiting the proliferation and migration of liver cancer cells (32). Elevated expression of MIR100HG has also been found to independently predict a poor prognosis in gastric cancer patients (33), while its upregulation in CRC has also been linked with a poor prognosis and enhanced invasive and migratory activity in tumor cells (34). We further found that PCED1B-AS1 and TRG-AS1 were involved in JAK-STAT signaling, Th17 cell differentiation, natural killer cell-mediated cytotoxicity, Th1 and Th2 cell differentiation, chemokine signaling, and T cell receptor signaling. PCED1B-AS1 was initially identified as an HIF-1α-dependent oncogenic lncRNA associated with glioma and with potential value as a prognostic biomarker and/or therapeutic target (35). There is also evidence that PCED1B-AS1 interacts with miR-194-5p to promote glioma cell survival and proliferation (36). TRG-AS1 expression was associated with the incidence of colon cancer (37) and has also been found to competitively bind to miR-877-5p and promote the proliferation of glioblastoma cells (38). While prior data pertaining to these four lncRNAs is limited, our immune-related lncRNA risk signature affirms their prognostic and functional relevance in patients with stage II–III CRC. LncRNA mainly acts by regulating mRNA expression through independent mechanisms, which the function is to encode protein. We mainly focus on the regulatory relationship between LncRNA and mRNA, so we only construct immune-related LncRNA-mRNA co-expression networks.

There are a few limitations to the present study. Most importantly, future research will be essential to understand the mechanisms whereby these four immune-related lncRNAs influence the survival of stage II–III CRC patients. Further prospective evaluation of their prognostic relevance in clinical trials will also be necessary to confirm their reliability as prognostic biomarkers. Despite these limitations, as we were able to independently validate the prognostic utility of the 4-lncRNA risk signature in three independent datasets, we believe it represents a powerful and promising tool for the evaluation of patients with stage II–III CRC.

Conclusions

We developed a novel immune-related lncRNA risk signature that could independently predict survival outcomes in stage II–III CRC patients. These findings offer a novel approach to evaluating CRC patients by potentially identifying novel lncRNA targets amenable to immunotherapeutic treatment in this disease.

Acknowledgements

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Footnote

Reporting Checklist: The authors have completed the STARD reporting checklist. Available at https://dx.doi.org/10.21037/jgo-21-594

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://dx.doi.org/10.21037/jgo-21-594). All authors report that this study was funded by the State Key Laboratory of Pathogen and Biosecurity (Academy of Military Medical Science) SKLPBS2138, and the Shenzhen Science and Technology Project (Project number: JCYJ20180507183842516). The authors have no other conflicts of interest to declare.

Ethical Statement: The authors are accountable for all

aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Institutional ethical approval and informed consent were waived.

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