Investigating the correlation between DNA methylation and immune-associated genes of lung adenocarcinoma based on a competing endogenous RNA network

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Abstract. In recent years, there have been major breakthroughs in immunotherapies for the treatment of cancer. However, different patients have different responses to immunotherapy. Numerous studies have shown that the accumulation of epigenetic abnormalities, such as DNA methylation, serve an important role in the immune response of lung adenocarcinoma (LUAD). To investigate the effects of DNA methylation on tumor immunity with survival and prognosis, relevant studies can be performed based on the regulatory mechanisms of RNA molecules. For example, long non-coding RNAs (lncRNAs), which regulate gene expression through epigenetic levels. By constructing an immune-associated competitive endogenous RNA (ceRNA) network, the present study identified the regulatory associations among 3 key immune-associations mRNAs, 2 microRNAs (miRs) and 29 lncRNAs that were closely associated with the prognosis of patients with LUAD. The molecular biology analysis indicated that hypomethylation of the 1101320-1104290 regions of chromosome 1 resulted in the low expression levels of *LINC00337* and that *LINC00337* may affect the expression levels of *CHEK1* by competitively binding with *human (has)-miR-373* and *hsa-miR-195*. Therefore, abnormal DNA methylation in lncRNA-associated regions caused their abnormal expression levels, which further affected the interactions between RNA molecules. The interactions between these RNA molecules may have regulatory effects on tumor immunity and the prognosis of patients with LUAD.

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Introduction

Studies have reported some key breakthroughs in tumor immunological therapies for lung cancer (1,2). For example, a blockade of immune checkpoint therapy regulated T cell activity to enhance the antibody-tumor immune responses. Significant advances in the molecular characterizations of lung cancer have led to the creation of effective immunotherapies that assist in the recognition of cancer as foreign by the host immune system, stimulate the immune system and relieve the inhibition that allows tumor growth and spread (3). However, immunotherapies have a number of limitations in clinical applications due to the existence of tumor heterogeneity and immunological suppression (4,5). In a previous study that focused on tumor immunological genomics, the assessment of tumor immune infiltrating cells served a key role in investigating the way in which the interactions between the tumor and the immune system affect patient outcome (6).

In an investigation into lung cancer and its immune regulations, a previous study demonstrated the significance of CpG islands in the lung immune responses of granulomatous lung diseases (7). Simultaneously, the study indicated that long non-coding RNAs (lncRNAs) serve an important role in directing the development of various immune cells and controlling dynamic transcriptional programs and were hallmarks of immune cell activation (7). In addition to the direct regulation of lncRNAs by DNA methylation via interactions with their promoter regions, previous studies have revealed several intricate regulatory associations between lncRNAs and DNA methylation (8,9). In the *cis*-methylated lncRNAs group, DNA methylation adjacent to a lncRNA locus, such as the promoter or the imprinting control region, directly modulate the expression levels of target lncRNAs as a *cis*-regulator (10). In *trans*-methylation due to lncRNAs, lncRNAs regulate the DNA methylation of a *trans*-genomic locus as an intermedium by recruiting DNA (cytosine-5-)-methyltransferase (11,12). In the *trans*-methylation-regulated lncRNAs group, an alteration in the DNA methylation state at a specific genomic locus regulates the transcription of its antisense-oriented lncRNAs (13,14). The lncRNA extracoding *CEBPA* transcribed from the

CEBPA gene locus is reported to be critical for the regulation of DNA methylation at this site through interactions with DNA methyltransferase 1 (15).

Based on the interactions between DNA methylation and lncRNAs, it is easy to investigate the role of methylation on the tumor immune responses from the perspective of lncRNAs. Currently, the competitive endogenous (ce)RNA hypothesis provides an approach to study the regulatory mechanisms of RNA molecules (16). ceRNA is an RNA with a binding site that competitively binds to a micro (mi)RNA to inhibit its target gene regulation (16). Pseudogenes, lncRNAs, miRNAs and other types of RNAs can be regulated by ceRNA mechanisms (7). Therefore, the present study investigated the roles of DNA methylation in tumor immune cell infiltration abundance and its association with patient prognosis from the novel perspective of RNA molecules, DNA methylation and their regulatory mechanisms.

The present study investigated the cause behind the differences in tumor immune responses from a new perspective. The present study investigated how DNA methylation affected the interactions between tumors and immune cells and between RNA molecules. Starting with DNA methylation, the present study revealed DNA methylation abnormalities, differentially expressed RNA (DERNA) molecules and mutual regulatory associations between DNA methylation and RNA molecules and assessed potential associations between DNA methylation and tumor immune infiltration in LUAD.

Materials and methods

Acquisition and preprocessing of the data. LUAD transcriptome, miRNA, DNA methylation and associated clinical data were downloaded from The Cancer Genome Atlas (TCGA) website (TCGA-LUAD; portal.gdc.cancer.gov/). The transcriptome data contained 594 samples (59 normal samples and 535 tumor samples), the miRNA data contained 577 samples (46 normal samples and 531 tumor samples) and the DNA methylation data included 507 samples (30 normal samples and 477 tumor samples). These data were downloaded on April 1, 2018. For the original data, most of the data with RNA expression level=0 were deleted. For the transcriptome data, the RSEM-preprocessed per million transcript method was used to isolate and obtain lncRNA and mRNA expression data (17).

Extraction of the differential methylation regions and associated lncRNAs. For the preprocessed DNA methylation data, differential DNA methylation regions and associated lncRNAs were identified using the lncDM algorithm in a package in R 3.5.3 in March 11, 2019. LncDM is a novel computational method for identifying differentially methylated regions in specific diseases. LncDM uses the Illumina HumanMethylation450 BeadChip GENCODE of all annotation information based on the reannotation method and obtains the CpG values of the differential transcript (18). The gene functional regions were used to calculate the patterns of differential methylation. The screening criteria for the differentially methylated regions were adjusted to $P<0.05$ and β diffcut >0.3. The β diffcut value was used to reflect the differential situations between samples.

Extraction of the abnormally expressed RNA molecules. For those RNA molecules in original data that were subjected to deletion processing, DERNA were screened using the DEseq2 algorithm in R software. The DEseq2 method uses shrinkage to estimate fold change, thus improving the stability and interpretability of the DEseq-based estimation (19). The screening criteria for the DERNA were $|\log FC|>2$ and $P<0.05$.

Selection of the immune cell-associated genes. To investigate the cause of tumor heterogeneity and its association with prognosis, the immune infiltration abundance of relevant LUAD tumor samples needed to be calculated. In this process, the TIMER 1.0 algorithm was used (20). First, the CHAT package was used to estimate the tumor purity of the samples (21), then the COMBAT package was used to combine all relevant immune cell gene expression data to eliminate the effect of batch processing of different data (22). Genes negatively correlated with tumor purity were selected and matched with some known immune cell (B cell, CD4+ and CD8+ T cell, neutrophil, macrophage and dendritic cell) tag genes to obtain 6 immune cell-associated genes.

Construction of the DNA methylation and immune-associated ceRNA network. To construct the ceRNA network, multiple databases were used to investigate the inter-relationships between DERNA molecules and DNA methylation. First, the differential methylation region (DMR)-DElncRNA relationship pairs were identified by employing the Lnc2Meth 1.0 (bio-bigdata.hrbmu.edu.cn/Lnc2Meth/) database (18). Next, the DElncRNA-DEmiRNA relationship pairs were identified by searching the miRcode 1.0 (mircode.org/) database (23). miRNAs were then annotated with 3p and 5p, which were used to distinguish between the 3' and 5' arms of miRNA precursors that target different sites, using the Starbase v2.0 (starbase.sysu.edu.cn/) database (24). Finally, the DEmiRNA-DEmRNA relationship pairs (25-27) were identified using miRDB v3.0 (mirdb.org/), miRTarBase 1.0 (mirtarbase.mbc.nctu.edu.tw/) and TargetScan v7.2 (targetscan.org/vert_72/). The visualization of this ceRNA network was achieved using Cytoscape version 3.6.0 software (28). In order to study the overall survival for the associated RNA molecules in the network, the present study used the Renyi test (29), a weighted test method, to generate survival curves.

Clinical analysis of the immune cell genes associated with DNA methylation. In order to investigate the effects of DNA methylation-associated tumor immune infiltration on clinical data, a multivariate regression model with associated factors, such as infiltration abundance, tumor purity, stage, infection status, age and sex was constructed. In this process, a single-factor Cox analysis was performed on the differential DNA methylation-associated immune genes. Those associated mRNAs with P-value <0.0001 were screened as clinically relevant genes. A multivariate risk regression analysis was then performed based on these clinically relevant genes. The theoretical basis of this model was the semiparametric regression model proposed by the British statistician D.R. Cox (30). The dependent variables of this model were the overall survival rates and result of survival. This model had the advantage of analyzing the influences of multiple factors on overall survival

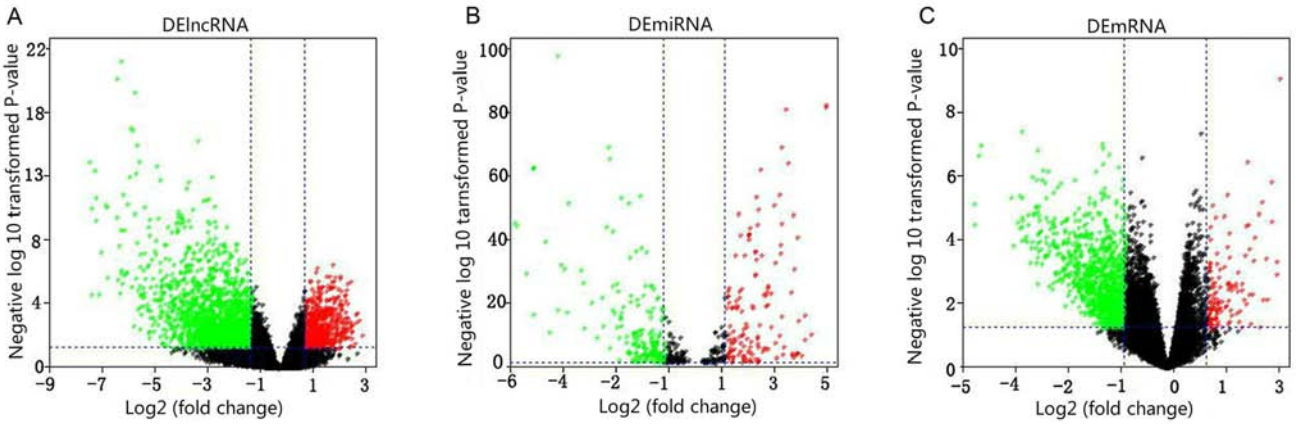

Figure 1. Volcano plots of the differentially expressed RNA molecules. The distributions of the differentially expressed in (A) lncRNAs, (B) miRNA and (C) mRNA. Red plots represent upregulated RNA molecules, green plots represent downregulated and the black plots represented no differences. lnc, long non-coding; mi, micro; DE, differentially expressed.

rates and the truncation analysis of overall survival rates simultaneously. Finally, Kaplan-Meier curves were used to verify the overall survival rates within 5 years. Kaplan-Meier curves were generated using the log-rank test. These aforementioned processes were completed with the survival package 3.2-3 of R software (CRAN.R-project.org/package=survival).

Functional enrichment analysis. For the immune genes associated with DNA methylation, the biological functions of genes were analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) functional enrichment (geneontology.org/). The KEGG (kegg.jp/) and GO functional enrichment analyses were performed by using the Database for Annotation, Visualization and Integrated Discovery (DAVID; v6.8) website (31).

Results

Extraction of DERNAs and differential DNA methylation regions. To understand the RNAs and methylation regions that have undergone significant changes in tumor and control tissues, differential data were first extracted. The extraction process of differential data involved extracting DERNAs, DNA methylation regions and the regulatory correlations between DNA methylation regions and lncRNAs. In the process of extraction, DERNA molecules in LUAD, such as DElncRNAs, DEmiRNAs and DEmRNAs were screened using the DEseq2 method in R software. The screening criteria for DERNA molecules were |log FC|>2 and P<0.05 (Fig. 1). A total of 1,187 DElncRNAs, 133 DEmiRNAs and 1,474 DEmRNAs were obtained. The extracted RNA molecules were increased or decreased expressed in the patients with LUAD. It can be inferred that the abnormal expression levels of RNA molecules were associated with LUAD.

The processed DNA methylation data were used to screen for differential DNA methylation regions and the corresponding lncRNAs using the lncDM method in R software. Those methylation regions were removed when methylation differences were <0.05. The detailed information regarding the DMRs included the methylation regions, the associated lncRNAs and the methylation difference are presented in Table SI. During the process, 1,692 DMRs associated with the lncRNAs were extracted. It was hypothesized that the increased or decreased expression levels of lncRNAs were associated with abnormal methylation.

Construction of the immune-associated ceRNA network. In order to investigate the regulatory mechanisms of gene expression associated with immune cells from the perspective of DNA methylation, a DNA methylation and immune-associated ceRNA network was constructed based on the internal competitive RNA hypothesis (Fig. 2). By matching the Lnc2Meth 1.0 database, 1,692 DMR-DElncRNA relationship pairs were identified, of which there were 64 lncRNAs. In addition, 285 DElncRNA-DEmiRNA relationship pairs were identified by matching the Micode 1.0 database, of which there were 21 miRNAs and 64 lncRNAs in DElncRNA-DEmiRNA relationship pairs. In addition, 1,177 DEmiRNA-DEmRNA relationship pairs were obtained by searching the databases of interactions between miRNAs and mRNAs, of which there were 21 miRNAs and 92 mRNAs.

Then, the RNA molecules in the aforementioned relationship pairs were matched with the increased or decreased expressed RNA molecules yielding abnormally expressed relationship pairs. The abnormal expression levels of RNA molecules in relationship pairs are presented in Tables SII, SIII and SIV. The expression levels of *CHEK1*, *SLC7A11* and *CCNE1* were much lower in LUAD samples compared with normal samples (Table SII). The expression levels of *hsa-miR-373* and *hsa-miR-195* were much lower in LUAD samples compared with normal samples (Table SIII). The expression levels of *LINC00377*, *LINC00473* and *LINC00355* were much lower in LUAD samples compared with normal samples (Table SIV). The changes in DNA methylation regions and corresponding RNAs of the ceRNA network are presented in Table SV.

According to the abnormally expressed relationship pairs, the ceRNA network was constructed. In order to improve the current understanding of the ceRNA network, the visualization of this ceRNA network was achieved using Cytoscape version 3.6.0 software (27). This network included 3 mRNAs, 2 miRNAs and 29 lncRNAs. It can be inferred that low expression levels of the lncRNA LINC00337 were associated with

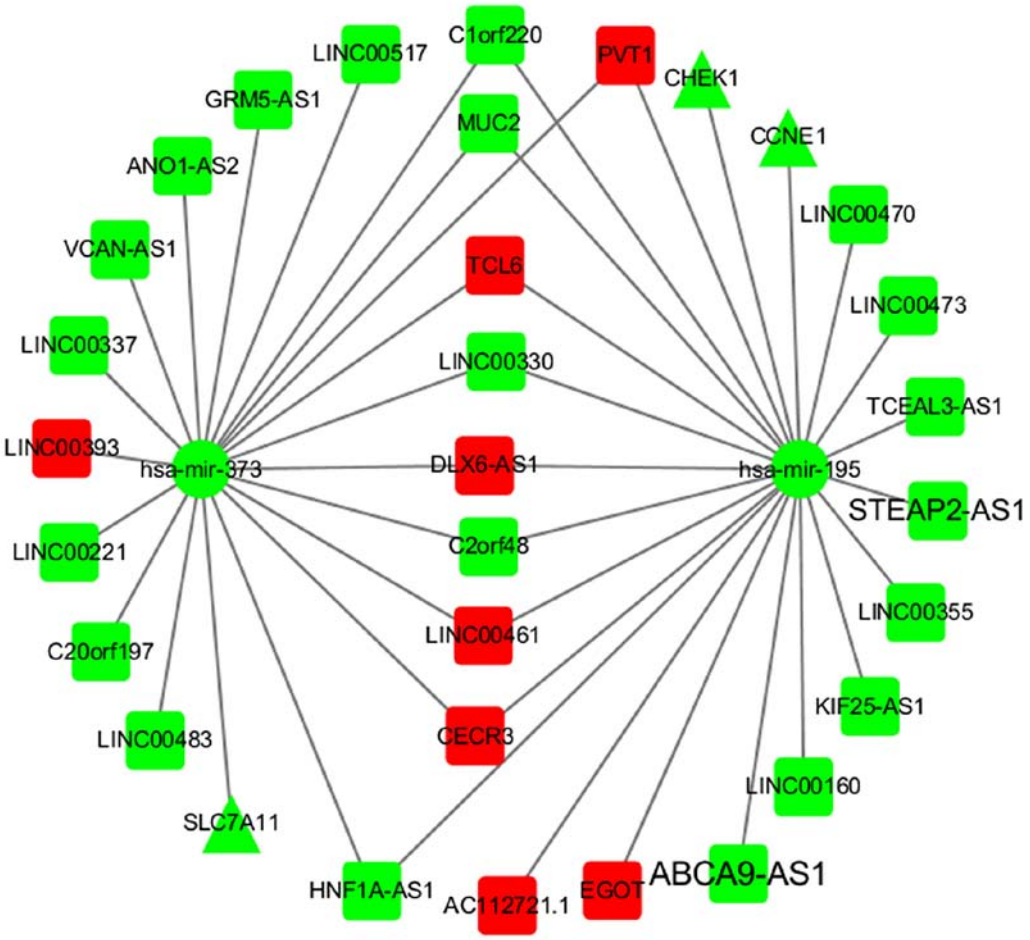

Figure 2. DNA methylation and immune-associated competing endogenous RNA network. Circles represent miRNAs, triangles represent mRNAs and rectangles represent lncRNAs. For lncRNAs, red rectangles represent lncRNAs associated with the DNA methylation regions that were hypermethylated, and green rectangles represented hypomethylation. For miRNAs and mRNAs, red represents downregulation and green represents upregulation. miR/miRNA, microRNA; hsa, *Homo sapiens* (human).

hypomethylation in the corresponding DNA regions (Fig. 2). The miRNAs (*hsa-miR-372*, *hsa-miR-373*) can bind to *mRNAs* (*SLC7A11*, *CHEK1* and *CCNE1*). The lncRNAs (*LINC00337* and *LINC00355*) may bind to miRNAs (Fig. 2).

The associated RNA molecules in this network were critical for investigating immunity and DNA methylation. The present study used the Renyi test (29), a weighted test method, to generate survival curves for the associated RNA molecules in the network (Fig. 3). In the present study, the Renyi test weight is 1.

The criterion for expression levels of RNA molecules was derived from the criteria in the premier increased or decreased expression level extraction of RNA molecules. The screening criteria for DERNA molecules were |log FC|>2 and P<0.05, which indicated that the expression levels of the RNAs were evidently increased or decreased in LUAD samples. Survival curves of all the RNA in the network were generated and survival curves with P<0.05 were selected as this indicated that abnormal RNA expression levels were significantly associated with survival rates in the LUAD samples.

The patients with LUAD that exhibited low expression levels of mRNA *CCNE1* (Fig. 3E), *CHEK1* (Fig. 3F) and *SLC7A11* (Fig. 3G) had improved survival rate. The patients with LUAD that exhibited high expression levels of *TCEAL3-AS1* (Fig. 3C) had improved survival time. The patients with LUAD that exhibited low expression levels of lncRNA *LINC00337* had improved survival rate (Fig. 3A). These results indicated that the increased or decreased expression levels of immune cells genes affected the survival rate of patients with LUAD and suggested that the interactions between DNA methylation and RNA molecules may affect the responses to immunotherapies. The associated mRNAs can be considered potential markers of immunotherapies in LUAD. It was also verified that the RNAs in the network influenced the survival rate of patients with LUAD.

In order to investigate the expressional relationships between the RNA molecules, correlational analyses of the potential mRNAs and lncRNAs in the network were performed. There may be several competitive relationships in these RNA molecules. The expression levels of *CHEK1* were positively correlated with all the associated lncRNA molecules including *LINC00473*, *LINC00337* and *LINC00355*. Among them, *CHEK1* was strongly correlated with *LINC00337* (Fig. 4A). There were also positive correlations between the expression levels of *CCNE1* and the majority of its associated lncRNAs, including *C20orf48*, *LINC00377* and *TCL6* (Fig. 4B). These results verified the hypothesis of the present study based on the ceRNA hypothesis. It can be inferred that *LINC00337* affects

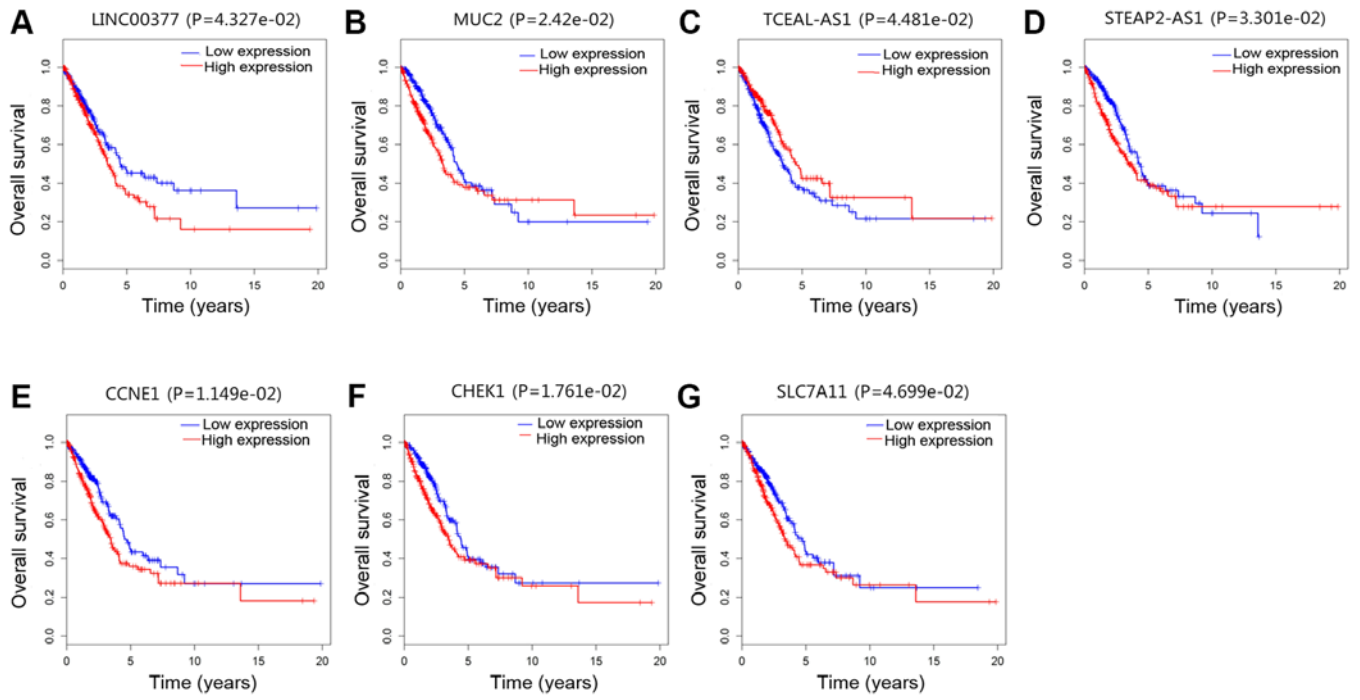

Figure 3. Survival curves of associated RNAs. The survival curves of (A) LINC00337, (B) MUC2, (C) TCEAL-AS1, (D) STEAP2-AS1, (E) CCNE1, (F) CHEK1 and (G) SLC7A11. The vertical axis is the overall survival rate of LUAD samples. The horizontal axis is the survival ratios in LUAD samples with differentially expressed RNA molecules. LUAD, lung adenocarcinoma.

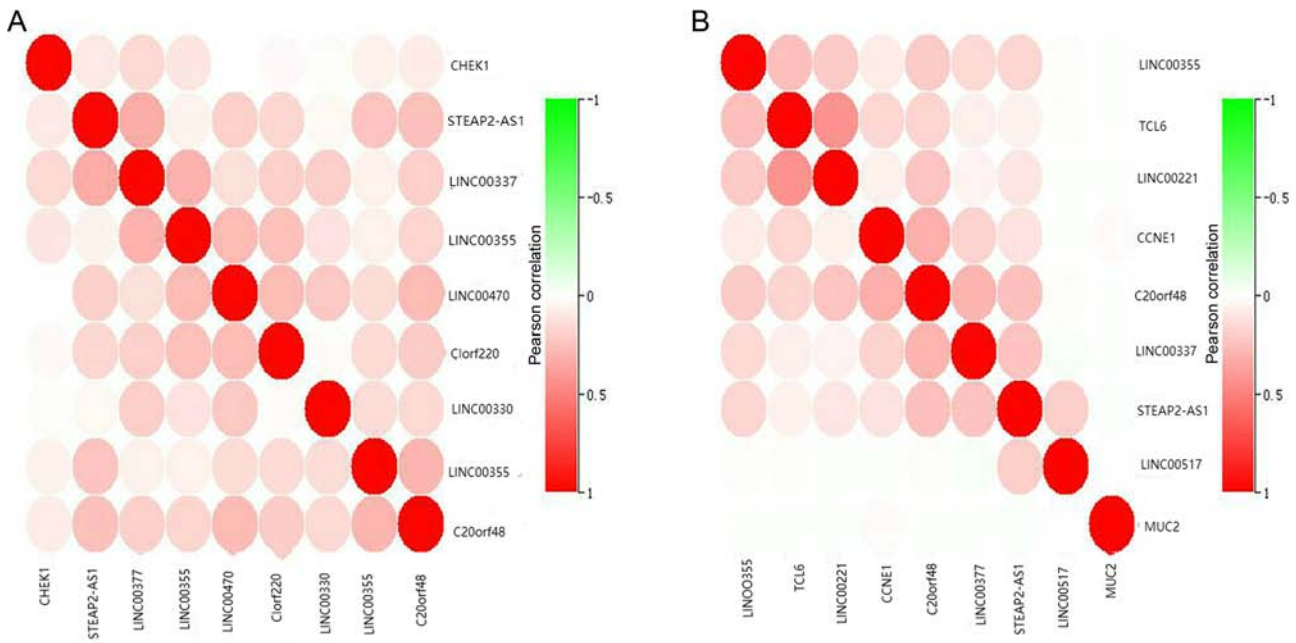

Figure 4. Expression correlations between mRNAs and associated lncRNAs. The expression correlations between associated lncRNAs and (A) mRNA CHEK1 and (B) mRNA CCNE1 were analyzed. The positive correlations were displayed in red. lnc, long non-coding.

the expression levels of CHEK1 by competing with the binding of *hsa-miR-373* and *hsa-miR-195* from the ceRNA network and competitive relationships. Concerning the changes in DNA methylation regions and the corresponding lncRNAs, it can be deduced that the hypomethylation of the 1101320-1104290 regions of chromosome 1 resulted in the low expression levels of *LINC00337*, and that *LINC00337* may have affected the expression levels of *CHEK1* by competitively binding with *hsa-miR-373* and *hsa-miR-195*, thereby affecting tumor immune cells and overall survival rates in patients with LUAD.

DNA methylation-associated immune genes analysis. In the process of constructing the ceRNA network, the correlations between RNA molecules were obtained by searching a large number of databases. In addition, 181 mRNAs associated

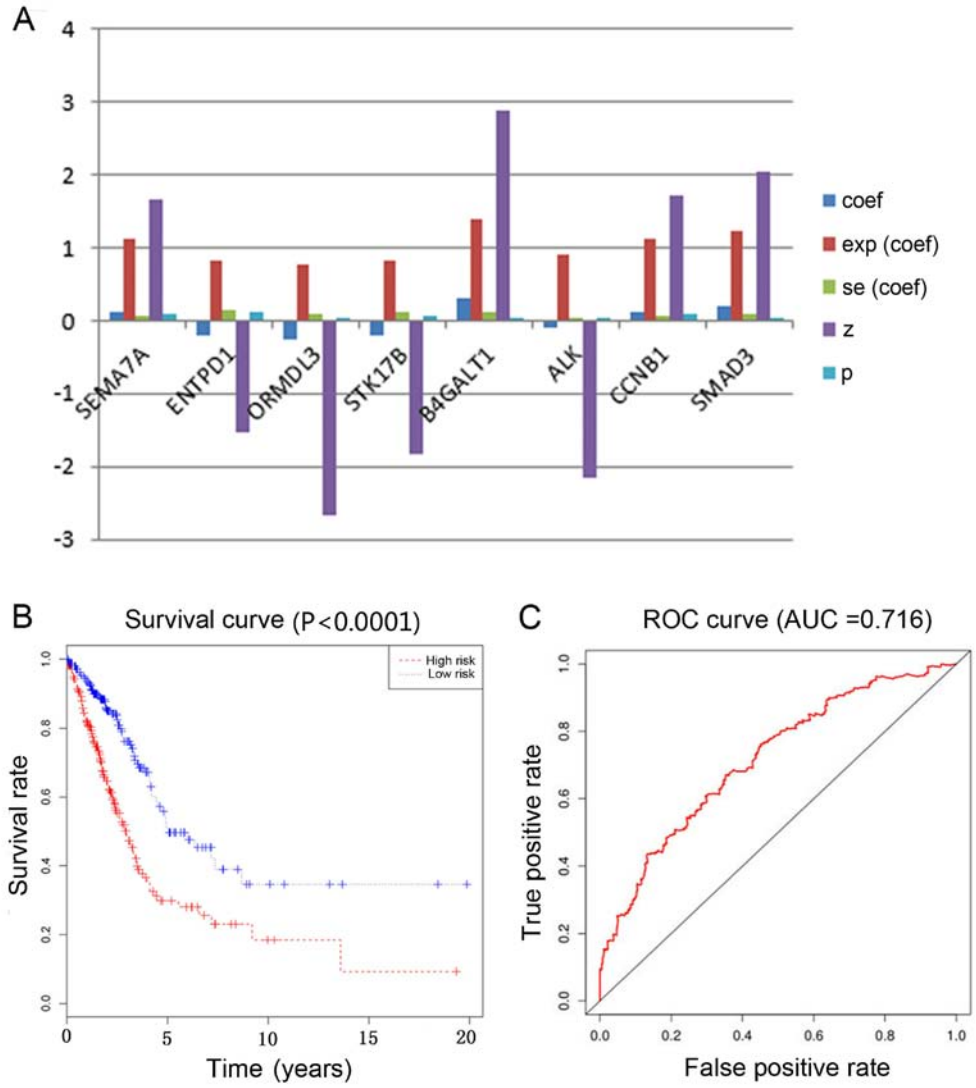

Figure 5. Cox regression model of genes associated with methylation and immunity. (A) Parameters of the model. The vertical axis is the selected mRNA molecules; the horizontal axis is the expression level-associated parameters of mRNA molecules. (B) The survival curves of high-risk and low-risk samples. The vertical axis represents the survival rates of the LUAD samples, the horizontal axis represents survival time of the LUAD samples. (C) The ROC curve in the model. LUAD, lung adenocarcinoma; coef, correlation coefficient; exp (coef), risk ratio; se (coef) standard error; z, Wald test statistic; P, probability; ROC, receiver operating characteristic.

with tumor immunity and clinical outcomes were identified. In order to investigate how these associated mRNAs influence survival rates in patients with LUAD, a multivariate Cox regression model was performed using 8 mRNA expression values (*SEMA7A*, *ENTPD1*, *ORMDL3*, *STK17B*, *B4GALT1*, *ALK*, *CCNB1*, *SMAD3*) to reflect the associations between gene expression levels and the survival rates in patients with LUAD (Fig. 5A). A survival rates analysis between the high-risk group and the low-risk group was conducted (Fig. 5B). The samples were divided based on the median value of the risk values. The risk value was derived from the multivariate Cox regression analysis. The cut-off value for high-risk samples was a risk value >0.956. Based on the survival curves between high-risk and low-risk patients, it can be seen that low-risk patients have improved overall survival rates. In the present study, the area of the ROC curves was 0.716 (Fig. 5C). The area of the ROC curve indicated the accuracy of diagnostic methods. The criterion of trustworthy accuracy was when the area of the ROC curves >0.7. The prediction of survival outcomes had reliable accuracy in patients with LUAD. The results demonstrated that the expression levels of the DNA methylation-associated immune cells genes affected the prognosis of patients with LUAD.

Finally, the functional enrichment analysis of DNA methylation-associated immune cells genes was conducted using the DAVID website. Some pathways were found to be associated with immune processes, including the pathway terms 'B cell receptor signaling pathway' and the 'T cell receptor signaling pathway'. Several biological processes involving immune processes were also obtained, including the GO terms 'Positive regulation of cell migration' and the 'T cell receptor signaling pathway' (Fig. 6). The results of the enrichment analysis also indicated that these associated genes served an important role in LUAD tumor immunity. From these results, it can be inferred that DNA methylation may have regulatory effects on the expression levels of the immune cells genes.

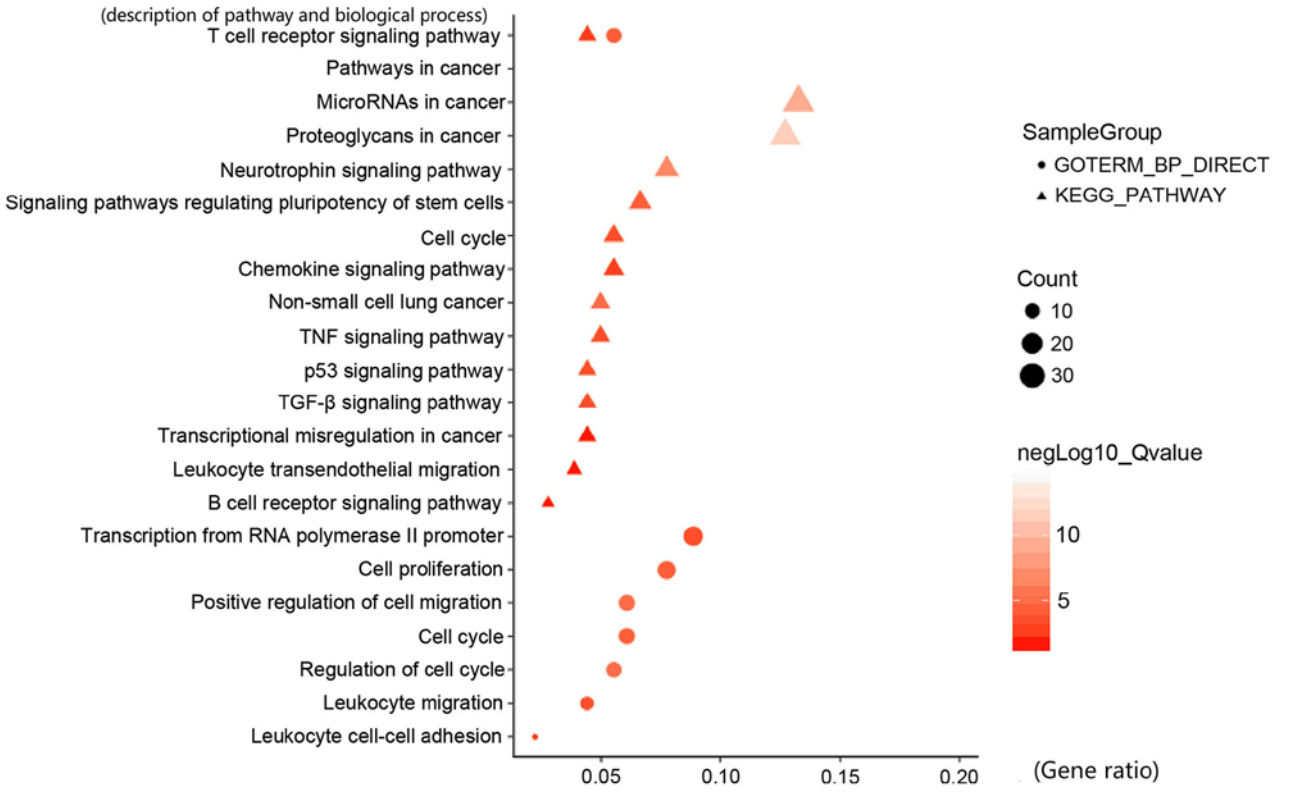

Figure 6. Functional enrichment analysis of genes associated with immune and DNA methylation. The horizontal axis is the ratios of genes, the vertical axis is the description of pathway and biological process. The shape of the symbols was used to distinguish pathways and biological process. The color of the symbols represents probability and the size of the symbols represented the counts of enriched genes. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Discussion

Understanding tumor immune cells infiltration is crucial for investigating the differences in responses to cancer immunotherapies and for developing effective immunotherapies. DNA methylation may explain the variabilities in immune cells of lung cancer in response to antigens (7). However, it is not completely clear how DNA methylation affected tumor immune cell infiltration. The present study investigated how DNA methylation affected the interactions between tumors and immune cells from the perspective of DNA methylation affecting the interactions between RNA molecules. A ceRNA network with associated DNA methylation and immune cell genes was constructed to study the associations between DNA methylation and the expression levels of immune cell genes.

In the present study, differential DNA methylation regions and DERNA molecules in lung adenocarcinoma (LUAD) samples were extracted. lncDM software (lncdm.r-forge.r-project.org/) was used to extract differential DNA methylation regions and their corresponding lncRNAs (18). The DEseq2 method was used to extract DElncRNAs, DEmiRNAs and DEmRNAs in LUAD samples (19). Then, to study the effects of differential DNA methylation on tumor immune infiltrating cells, tumor immune cells infiltration abundance was calculated. When calculating the infiltration abundance of tumor immune cells, CIBERSORTx (cibersortx.stanford.edu/) outperformed other algorithms in eliminating noise, estimating unknown mixture contents and accuracy. However, the algorithm was prone to bias due to the statistical collinear effect generated by the regression analysis, whereas the TIMER 1.0 algorithm (cistrome.dfci.harvard.edu/TIMER/) blocked immunological marker genes and highly expressed genes to eliminate bias effects and collinearity between immune cells, ensuring the accuracy of reasoning (20). Thus, the TIMER 1.0 algorithm was applied in the present study. Databases of molecular associations between RNAs (Lnc2Meth 1.0, miRcode 1.0, Starbase v2.0, miRDB v3.0, miRTarBase 1.0 and TargetScan v7.2) were used to investigate the associations between RNA molecules and differential DNA methylation regions (13,23-27). Based on the ceRNA hypothesis, a ceRNA regulatory network of immune genes was established to determine the regulatory associations between DNA methylation and RNA molecules in LUAD. By using mRNAs expressional data and clinical data associated with differential DNA methylation regions, a regression model was constructed to investigate the associations between methylation-associated tumor immune cells infiltration and overall survival rates (30).

Compared with previous studies (6-8) that focus on the tumor immune cells infiltration, the present study further analyzed the regulatory roles in it. It was hypothesized that the hypomethylation of the 1101320-1104290 regions of chromosome 1 may affect the expression levels of *CHEK1*, thereby affecting tumor immune cells and survival outcomes in patients with LUAD. The ceRNA network was further enriched from the perspective of DNA methylation. From the present study, it can be deduced that the hypomethylation of the 1101320-1104290 regions of chromosome 1 resulted in the low expression levels of *LINC00337*, and that *LINC00337*

may affect the expression levels of *CHEK1* by competitively binding with *hsa-miR-373* and *hsa-miR-195*.

The present study was performed based on theoretical research described in previous studies (7-9,16). There is evidence that lncRNAs play important roles in directing the development of various immune cells and controlling dynamic transcriptional programs, which are hallmarks of immune cell activation (7). Additionally, a ceRNA is an RNA with an miRNA binding site that competitively binds to an miRNA to inhibit its target gene regulation (16). Pseudogenes, lncRNAs, miRNAs and other types of RNA, can be regulated by ceRNA mechanisms (7). Therefore, the ceRNA hypothesis provided an approach to investigate the regulatory mechanisms of RNA molecules. In addition, it was possible to construct a ceRNA network using existing databases of the interactions between DNA methylation and RNA molecules. In addition, previous studies have shown that DNA methylation directly regulates lncRNAs through interactions with their promoter regions (8,9). For example, in the *cis*-methylated lncRNAs group, DNA methylation adjacent to an lncRNA locus, such as the promoter or the imprinting control region, directly modulates the expression levels of target lncRNAs as a *cis*-regulator (9).

DERNA molecules and DEDNA methylation regions associated with the lncRNAs were extracted in the present study to understand the RNAs and methylation regions that have undergone significant changes in tumor and control tissues. In order to investigate the regulatory mechanisms of genes expression levels associated with immune cells from the perspective of DNA methylation, a DNA methylation and immune-associated ceRNA network was constructed based on the internal competitive RNA hypothesis. Next, the associations between the expression levels of RNA molecules in the network and the survival rates of patients were investigated. Then, correlational analyses of potential lncRNA-mRNA pairs in LUAD was conducted to further investigate the competitive relationships. Subsequently, how these associated genes affected survival rates was investigated in the present study, and analyses of the DNA methylation-associated immune cells genes were performed. Finally, enrichment analyses of DNA methylation-associated immune cells genes were performed.

According to the analysis of the results, it can be inferred that hypomethylation of the 1101320-1104290 regions of chromosome 1 resulted in the low expression levels of *LINC00337*, and that *LINC00337* may affect the expression levels of CHEK1 by competitively binding with *hsa-miR-373* and *hsa-miR-195*, thereby affecting tumor immune cells and survival outcomes in patients with LUAD.

To verify the hypothesis, several studies (32-42) on the RNA molecules in the ceRNA network were searched. Previous studies (32-39) have also indicated that these RNAs showed important correlations in cancer, tumor immunity and DNA methylation. For example, *CCNE1* is significantly associated with TRIM58 methylation and the treatment of lung squamous cell carcinoma and may be used as a potential prognostic biomarker (32). Previous studies (33-35) have also shown that *CCNE1* is the most common amplified gene in lung cancer, and that its gene amplification was a therapeutic target for survival and lung cancer. Therefore, *CCNE1*-targeted therapy may be beneficial for *CCNE1* amplification in patients with lung cancer (33). *HOXC13*, which is directly targeted by miR-141, is highly expressed in LUAD and promotes the proliferation of LUAD by modulating the expression of *CCNE1* (34). Cyclin E1, encoded by the *CCNE1* gene, promotes G_1/S phase transition and chromosome instability (35). Cyclin E1 is downregulated by both *miR-497* and *miR-34a*, which synergistically retard the growth of human lung cancer cells (35).

In addition, smoking can induce the high expression levels of *SLC7A11* in oral cancer cells, which suggested that the expression levels of *SLC7A11* may encourage lung tumor progression (36-38). In addition, a previous study (36) demonstrated that *SLC7A11* regulates metabolic requirements during lung cancer progression and is a potential therapeutic target in non-small cell lung cancer. The transporter gene *SLC7A11* exhibits significant changes in enhancer DNA methylation and gene expression levels during pregnancy (37). In the present study, marker *SLC7A11* expression levels were determined in lung cancer cell lines using microarray data and Kaplan-Meier survival analyses were performed for each marker using patient clinical data (38). High expression levels of the marker *SLC7A11* are significantly associated with worse survival rates (38).

In a previous study (39), among the five associated pathways in LUAD, the p53 signaling pathway was the most significant, with *CHEK1* being identified as a hub gene. Previous studies (39-43) have also shown that miRNAs (*hsa-miR-195* and *hsa-miR-372*) in the network play important roles in tumor immunity and DNA methylation. For example, *hsa-miR-195* suppresses tumor growth and is associated with improved survival outcomes in several types of malignancy, including non-small cell lung cancer. The expression of hsa-miR-195 is lower in tumor tissues and is associated with poor survival outcomes (39). The overexpression of *miR-195* suppresses tumor cell growth, migration and invasion. *CHEK1* is a direct target of *miR-195*, which decreases the expression levels of *CHEK1* in lung cancer cells. Therefore, the high expression levels of *CHEK1* in lung tumors are associated with poor overall survival rates (39).

In addition, *hsa-miR-195* suppresses non-small lung cell cancer and is a predictive factor for lung cancer prognosis (39). A previous study (40) demonstrated that the expression levels of *hsa-miR-195* and O-6-methylguanine-DNA methyltransferase methylation status are associated with the clinical outcomes of the primary malignancy (40). In addition, *miR-372-3p* enhances the proliferation and invasion of lung squamous cell carcinoma cells by inhibiting *FGF9* (41). A previous study (42) conducted a comparative proteomic analysis of non-small-cell lung carcinoma (NSCLC) CL1-0 cells expressing *miRNA-372* and/or a vector only using two-dimensional gel electrophoresis, two-dimensional difference gel electrophoresis and liquid chromatography tandem mass spectrometry. Proteins identified as up- or down-regulated were further classified according to their biological functions (42). A number of the proteins identified in the study (42) may be potential diagnostic biomarkers of NSCLC, particularly phosphorylated *eIF4A-I*. In the corresponding study (43), to clarify the molecular mechanisms underlying the tumor suppressive roles of propofol in the human lung cancer cell line A549, the study detected the expression levels of *miR-372* and analyzed the mediating effects of *miR-372* on

the proliferation and metastasis of A549 cells (43). A previous study demonstrated that the expression levels of *LINC00473* can serve as a robust biomarker for the tumor LKB1 functional status that can be integrated into clinical trials for patient selection and treatment evaluation, and implicated *LINC00473* as a therapeutic target for LKB1-inactivated NSCLC (44).

The present study was primarily focused on the biological information analyses section, and so all the results were from the biological information analyses without biological verifications; for example, the direct validation experiments of the associations between DNA methylation and the expression levels of immune genes, the direct validation experiments of the associations between DNA methylation and tumor immune cells and survival outcomes in patients with LUAD. However, in order to verify the hypothesis of the present study, relevant studies were searched and some analyses were added in this discussion section.

The present study investigated the way in which DNA methylation affects the reactions between tumors and immune cells by constructing a ceRNA network. To further determine how the DNA methylation regions influenced the expression levels of immune genes by regulating the ceRNA network, the associations between the expression levels of RNAs, the overall survival rates of patients with LUAD and the competitive relationships between RNAs were investigated. Finally, a multivariate Cox regression model was constructed using 8 mRNAs expression values to investigate how these associated genes affected survival in patients with LUAD. The results revealed that hypomethylation of the 1101320-1104290 regions of chromosome 1 resulted in the low expression levels of *LINC00337* and that *LINC00337* may affect the expression levels of *CHEK1* by competitively binding with *hsa-miR-373* and *hsa-miR-195*, thereby affecting tumor immune cells and survival outcomes in patients with LUAD. The RNA molecules in the ceRNA network served important roles in DNA methylation and immunotherapies and can be used as biomarkers of immunotherapies in patients with LUAD. The results of the present study may provide a new perspective for the exploration of cancer-immune interactions.

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Availability of data and materials

LUAD transcriptome, miRNA, DNA methylation data and associated clinical data can be downloaded from The Cancer Genome Atlas (TCGA) website (TCGA-LUAD; portal.gdc.cancer.gov/).

Authors' contributions

CC, WK, XM and SW conceived and designed the present study. CC and WK conducted analyses. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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