

Long non-coding RNA expression identified by microarray analysis: Candidate biomarkers in human acral lentiginous melanoma

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Received March 20, 2019; Accepted November 14, 2019

DOI: 10.3892/ol.2019.11207

Abstract. Melanoma is a rare but fatal form of skin cancer and acral lentiginous melanoma (ALM) is one of its most common types. Long non-coding RNA (lncRNA) has emerged as a crucial molecule in the development and progression of human cancers, and several studies have revealed that lncRNAs may be associated with the pathogenesis, progression and metastasis of melanoma. To demonstrate the association between ALM and lncRNAs, microarray analysis was performed in tumor and adjacent non-tumor tissues. A total of 4,488 lncRNAs and 3,913 mRNAs were identified to be differentially expressed in these samples. Among them, 2,211 and 2,277 lncRNAs were upregulated and downregulated in the ALM samples compared with adjacent tissues, respectively. In addition, 1,191 and 2,722 mRNAs were upregulated and downregulated, respectively. Additionally, five randomly selected lncRNAs (fold-change >2; P<0.05) were validated by reverse transcription-quantitative PCR. An lncRNA and mRNA co-expression network and competing endogenous network analysis were also constructed. In summary, the results of the present study may reveal a novel mechanism associated with the pathogenesis and malignant biological processes of ALM and indicate that lncRNAs may serve as potential targets for the treatment of ALM.

Introduction

Melanoma is a rare, fatal type of skin tumor, which comprises four main types: Lentigo maligna melanoma, superficial spreading melanoma (SSM), nodular melanoma and acral lentiginous melanoma (ALM) (1). ALM, which generally affects the palms and soles of patients, has a low incidence in the Caucasian population and occurs mainly in patients of an Asian and African descent; up to 75% of all patients with melanoma have ALM (1). Patients with ALM usually have a poor prognosis due to difficulties in diagnosis and ALM tends to be identified at an advanced clinical stage or with high Breslow thickness (1-4). Genomic instability and poor response to biological agents in ALM also contribute to the poor outcome. Unlike SSM, in which BRAF mutation is the most observed aberration, KIT proto-oncogene receptor tyrosine kinase is the most mutated gene in ALM; however, this has only been identified in 15% of patients (5). Therefore, identification of more specific biomarkers for ALM is necessary.

Long non-coding RNAs (lncRNAs) have been demonstrated to serve crucial roles in tumorigenesis by diverse mechanisms and at various levels; for example, lncRNAs can act as mediators to regulate gene expression, combine with proteins to form a ribonucleoprotein complex and modify histones, recruit enzymes to regulate proximal or distant genes or serve as a decoy for transcription factors (6,7). Although previous studies (8-21) have reported that lncRNAs including HOTAIR, MALAT1, BANC1, ANRIL, SPRY4-IT1, LINC23, UCA1, SLNCR1 and SAMMSON served oncogenic functions in the progression and metastasis of melanoma, no studies are currently available on lncRNAs specifically related to ALM, and the mechanisms of lncRNA activity in ALM are still unclear. Therefore, identification of lncRNAs in ALM may provide value for early diagnosis and improved prognosis.

The present study aimed to investigate the role of lncRNAs in the pathogenesis of ALM by performing microarray analysis of the expression patterns of lncRNAs. This study may help to clarify the function of lncRNAs in ALM and provide evidence of their therapeutic and prognostic value.

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Key words: acral lentiginous melanoma, long non-coding RNA, microarray analysis, co-expression network, competing endogenous RNA network

Materials and methods

Tissue collection. A total of 12 samples, including six tumor and six adjacent non-tumor tissues, were collected in pairs from six patients with ALM (patient 1, male, 71 years; patient 2, male, 72 years; patient 3, female, 44 years; patient 4, female, 66 years; female 5, female, 74 years and patient 6, male, 55 years) between January 2017 and May 2018 at the Institute of Dermatology, Chinese Academy of Medical Sciences and Peking Union Medical College (Nanjing, China). The samples were immediately stored at -80°C . The study was approved by the Ethics Committee of the Institute of Dermatology, Chinese Academy of Medical Sciences and Peking Union Medical College (approval no. 2013-LC/KY-033). All participating patients gave informed consent.

RNA extraction and quality control. According to the manufacturer's protocol, total RNA was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). RNA quantity and quality were measured by NanoDrop ND-1000. Standard denaturing agarose gel electrophoresis (1%) or Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.) was used to assess integrity of RNA.

Microarray analysis. A total of 6 pairs of ALM and adjacent non-tumor tissues were used for the microarray assay to determine differentially expressed lncRNAs and mRNAs. Sample labeling and array hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology, Inc.). Arraystar Human lncRNA Microarray V4.0, designed for the global profiling of human lncRNAs and protein-coding transcripts, was used. The hybridized arrays were washed and then scanned using Agilent Scanner G2505C (Agilent Technology, Inc.). The acquired array images were analyzed using Agilent Feature Extraction software (version 11.0.1.1). Quantile normalization and subsequent data processing were performed using the GeneSpring GX v12.1 software package (Agilent Technologies, Inc.). Following quantile normalization of the raw data, lncRNAs and mRNAs that had flags in Present or Marginal ('All Targets Value') in ≥ 5 samples were selected for further analysis. Differentially expressed lncRNAs and mRNAs between cancerous and adjacent tissues were identified using the thresholds $P < 0.05$ and fold-change > 2 .

Gene ontology (GO) enrichment and kyoto encyclopedia of genes and genomes (KEGG) analysis. GO analysis (<http://www.geneontology.org>) was performed to explore the 'biological processes', 'cellular components' and 'molecular functions' of the identified differentially expressed mRNAs, and this was performed via top GO package in R environment. Using hyper-geometric distribution, which was made using R language, the significance between differentially expressed genes and KEGG could be observed. Pathway analysis was performed using KEGG, Biocarta and Reactome (<http://www.genome.jp/kegg/>). Fisher's exact test was used to find out if the overlaps between the DE gene list and the GO annotation list were greater than what was expected by chance. The significance of each GO term and pathway association are reflected by the P-value, and $P < 0.05$ was considered to

indicate a statistically significant result. The $-\log_{10}(P)$ was used to determine the enrichment of each GO term in the differentially expressed genes and the significance of the pathway associations. A lower P-value was considered to indicate a more significant correlation. The top 10 terms of GO analysis and KEGG analysis were all characterized by $P < 0.05$ and false discovery rate (FDR) < 0.05 .

Reverse transcription-quantitative (RT-q)PCR validation. A total of five randomly selected lncRNAs (fold-change > 2 , $P < 0.05$) were validated by RT-qPCR. These five lncRNAs belonged to the top 40 according to FC and its $P < 0.05$. All these lncRNA levels were gold level. Gold level meant that these selected lncRNAs had been validated by specific experiments and had relevant annotation, such as transcription units, function mechanisms and subcellular localization. Following RNA extraction, SuperScript™ III Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) was used to synthesize cDNA according to the manufacturer's protocol. Briefly, 2 μg RNA was mixed with 1 μl lncRNA specific primer Mix, 1.6 μl dNTP Mix, and RNase free water was added to a total volume of 13.5 μl . The sample was placed in a water bath for 5 min at 65°C and on ice for 2 min. Subsequently, 4 μl 5X First-Strand Buffer, 1 μl of 0.1 M DTT, 0.5 μl RNase Inhibitor and 1 μl SuperScript III RT were added. The reaction was performed for 1 min at 37°C , for 60 min at 50°C and for 15 min at 70°C . cDNA was eventually stored at -20°C . PCR was performed using ViiA 7 Real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) in a total volume of 10 μl , including 0.5 μl forward primer (10 μM), 0.5 μl reverse primer (10 μM), 2 μl cDNA, 5 μl 2X Master Mix and 2 μl double-distilled water. β -actin was used as an internal control. Primer sequences were as follows: β -actin forward, 5'-GTG GCGAGGACTTTTGATTG-3' and reverse, 5'-CCTGTAACA ACGCATCTCATATT-3'; NR_004845 forward, 5'-TTGGCA TACAGGTCTTTGTAGAT-3' and reverse, 5'-TTGGCATA CAGGTCTTTGTAGAT-3'; NR_026983 forward, 5'-ATCCCT GGTATTGAAGAGGTG-3' and reverse, 5'-AGATTGTTT GGGCAGTGTTAG-3'; NR_034040 forward, 5'-TGACAT CCGAATGCCATCCT-3' and reverse, 5'-GCTGCTGACAAA CAACCTGCT-3'; NR_036580 forward, 5'-AGCCCAGAT TCTCCTACCAGC-3' and reverse, 5'-CCTTCAGGTGGC TGTTTTGTAGT-3'; NR_037877 forward, 5'-ATGTTGACC ATGCAGCCAATT-3' and reverse, 5'-GTGTTTATCAGA GGTCATTTCCG-3'. The thermocycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 60 sec. The gene expression levels in tumor tissue relative to adjacent tissue were calculated as fold-change using the standard curve method (22). An unpaired t-test was used and $P < 0.05$ was considered to indicate a statistically significant difference.

lncRNA and mRNA co-expression (CNC) network. Using the validated lncRNAs and their target mRNAs, the CNC network was constructed by Cytoscape software (version 2.8.3; The Cytoscape Consortium) with the criterion that the Pearson correlation coefficient (PCC) of the lncRNA and mRNA correlation analysis was ≥ 0.95 . Additionally, $PCC \geq 0.95$ was considered to indicate meaningfully related pair. While performing CNC analysis and calculating PCC, the P-value

and FDR were also obtained ($P < 0.05$ and $FDR < 0.05$), which further confirmed the reliability of PCC.

Competing endogenous network analysis. Based on the hypothesis that RNA transcripts can crosstalk by competing for common microRNAs (miRNAs) and that miRNA response elements (MREs), which were the foundation of this interaction, the competing endogenous network was predicted. The prediction of such lncRNA-miRNA-mRNA interaction was based on the selected lncRNAs and related mRNAs. lncRNA/miRNA interactions were predicted by miRcode (<http://www.mircode.org>) and miRNA/mRNA interactions were predicted by miRanda (<http://www.miranda.org>) and TargetScan (<http://www.targetscan.org>).

Results

Differentially expressed lncRNA and mRNA profiles detected by microarray. According to the microarray expression profiling data and the filtering analysis including fold-change ≥ 2 and $P < 0.05$, a total of 4,490 lncRNAs and 3,915 mRNAs were identified to be differentially expressed between the ALM and adjacent non-tumor samples. Among them, 2,211 and 2,277 lncRNAs were upregulated and downregulated in the ALM samples compared with adjacent sections, respectively. In addition, 1,191 and 2,722 mRNAs were upregulated and downregulated, respectively. The top 5 upregulated lncRNAs were T380070, ENST00000554431, T097678, GSE61474_TCONS_00183926 and NR_015399, and the top 5 downregulated lncRNAs were TCONS_00013495, T367528, ENST00000598924, T050010 and TCONS_00010140. The top 5 upregulated mRNAs were ENST00000370287, NM_005367, NM_001129826, NM_001922 and NM_000273, and the top 5 downregulated mRNAs were ENST00000433840, NM_206998, NM_014867, NM_173595 and NM_004202. The variations in the lncRNA (Fig. 1A) and mRNA (Fig. 1B) expression profiles between ALM and adjacent tissue samples were assessed by scatterplot analysis, and volcano plots were constructed to demonstrate the association between the fold-changes and the statistical significance of the differentially expressed lncRNAs (Fig. 2A) and mRNAs (Fig. 2B). The expression patterns of lncRNAs and mRNAs were also demonstrated in hierarchical clustering (Fig. 3).

GO enrichment and KEGG pathway analysis. As lncRNAs affected coding gene expression by influencing mRNAs, GO enrichment analysis of significantly differentially expressed mRNAs was performed to determine the effects of these lncRNAs. GO categories of 'biological process' (BP), 'cellular component' (CC) and 'molecular function' (MF) were analyzed to determine the gene and gene product enrichment. The results of GO enrichment analysis are presented in Fig. 4. The analysis revealed that the majority of the BPs associated with upregulated mRNAs were involved in the events of pigmentation and melanocytes, such as 'developmental pigmentation', 'melanocyte differentiation', 'pigmentation' and 'melanin metabolite biosynthetic process' (Fig. 4A). The majority of the CCs of the upregulated mRNAs were associated with 'melanosome membrane', 'pigment granule' and 'melanosome' (Fig. 4B). Terms associated with channel activity were enriched in the

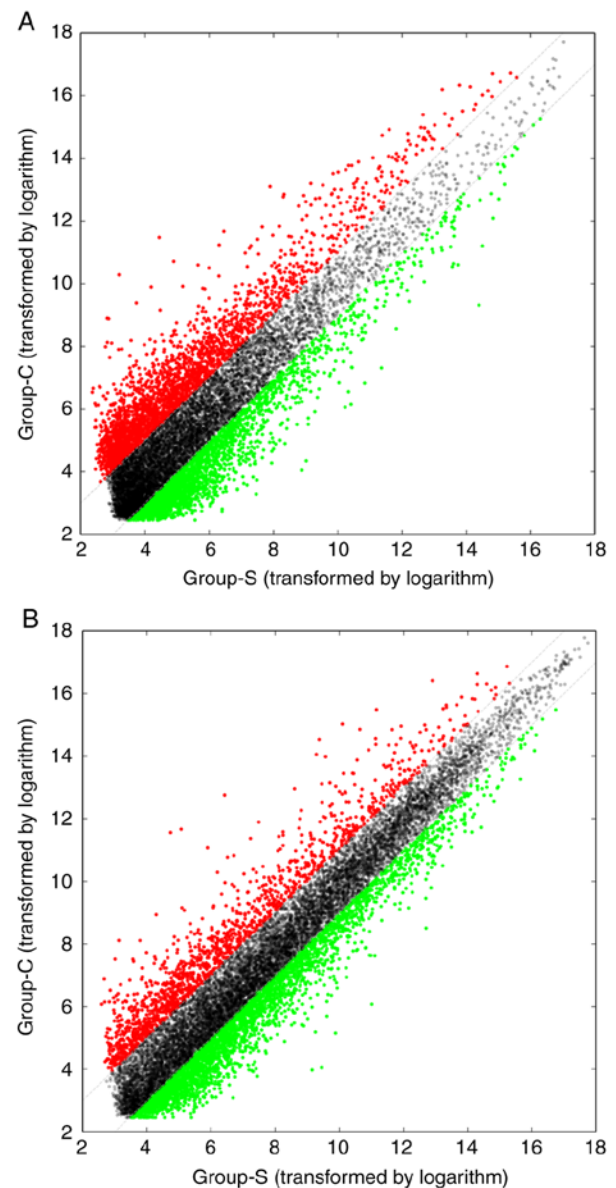


Figure 1. lncRNA and mRNA expression in ALM. (A and B) Scatterplots of (A) lncRNA and (B) mRNA expression variations between ALM and adjacent non-tumor tissues. Data points above the top line and below the bottom line indicate the lncRNA and mRNAs with fold-change > 2 in expression between the two groups. Red points represent upregulation of differentially expressed lncRNAs and mRNAs. Green points represent downregulation of differentially expressed lncRNAs and mRNA. Black points represent none-differentially expressed lncRNAs and mRNAs. The values of the x and y axes show the averaged normalized signal values of the group (log₂ scaled). Group-S, adjacent non-tumor tissues; group-C, cancerous tissues; lncRNA, long non-coding RNA; ALM, acral lentiginous melanoma.

MF category, including 'channel activity', 'cation channel activity' and 'calcium channel activity' (Fig. 4C). For the downregulated mRNAs, the majority of the enriched terms were associated with the cell junction and tissue development (Fig. 4D-F). For example, the top 3 terms in BP were 'tissue development', 'epidermis development' and 'epithelium development'. 'Cell-cell junction', 'anchoring junction' and 'cell-cell adhesion junction' were the top three terms in CC, whereas 'cell adhesion molecule binding', 'protein binding involved in cell-cell adhesion', 'cadherin binding' and 'kinase binding or actin binding' were the most three enriched terms in MF.

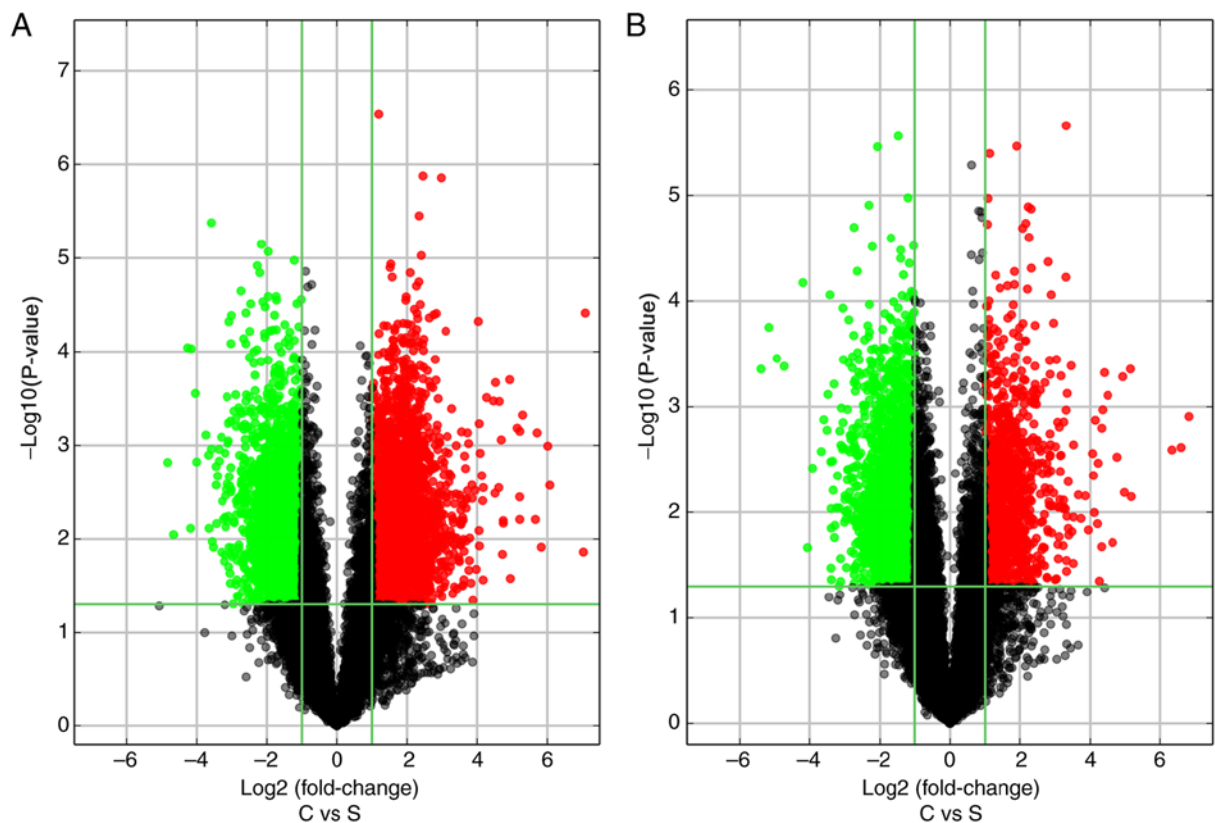


Figure 2. Volcano plots. (A and B) Differential expression of (A) lncRNAs and (B) mRNAs. Red points represent upregulation of differentially expressed lncRNAs and mRNAs. Green points represent downregulation of differentially expressed lncRNAs and mRNA. Black points represent none-differentially expressed lncRNAs and mRNAs. Two vertical lines represent the filtering standard of fold-change ≥ 2 . The term C vs. S at the bottom refers to acral lentiginous melanoma vs. adjacent tissues. Group-S, adjacent non-tumor tissues; group-C, cancerous tissues; lncRNA, long non-coding RNA.

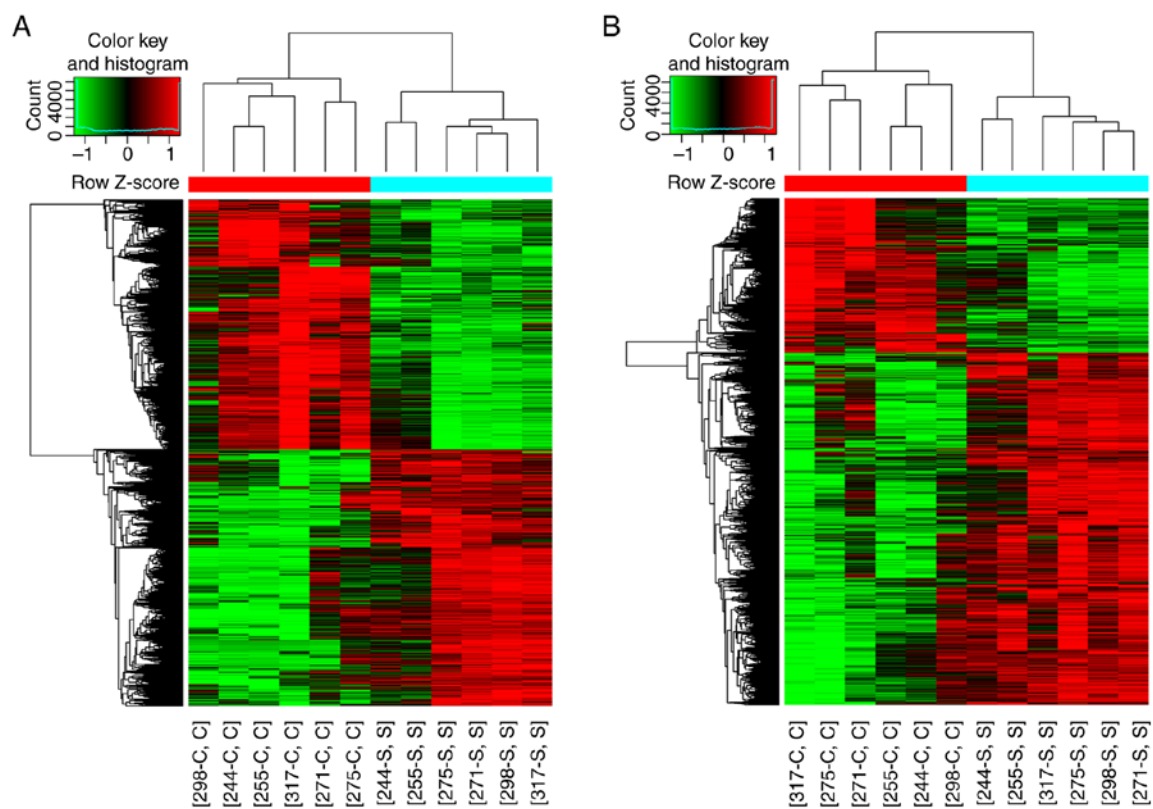


Figure 3. Hierarchical clustering. (A) lncRNAs and (B) mRNAs differentially expressed in acral lentiginous melanoma and adjacent non-tumor tissues were subjected to hierarchical clustering. Samples were divided into two groups by expression level; red indicates high relative expression and green indicates low relative expression. C, acral lentiginous melanoma tissues; S, adjacent tissues; lncRNA, long non-coding RNA.

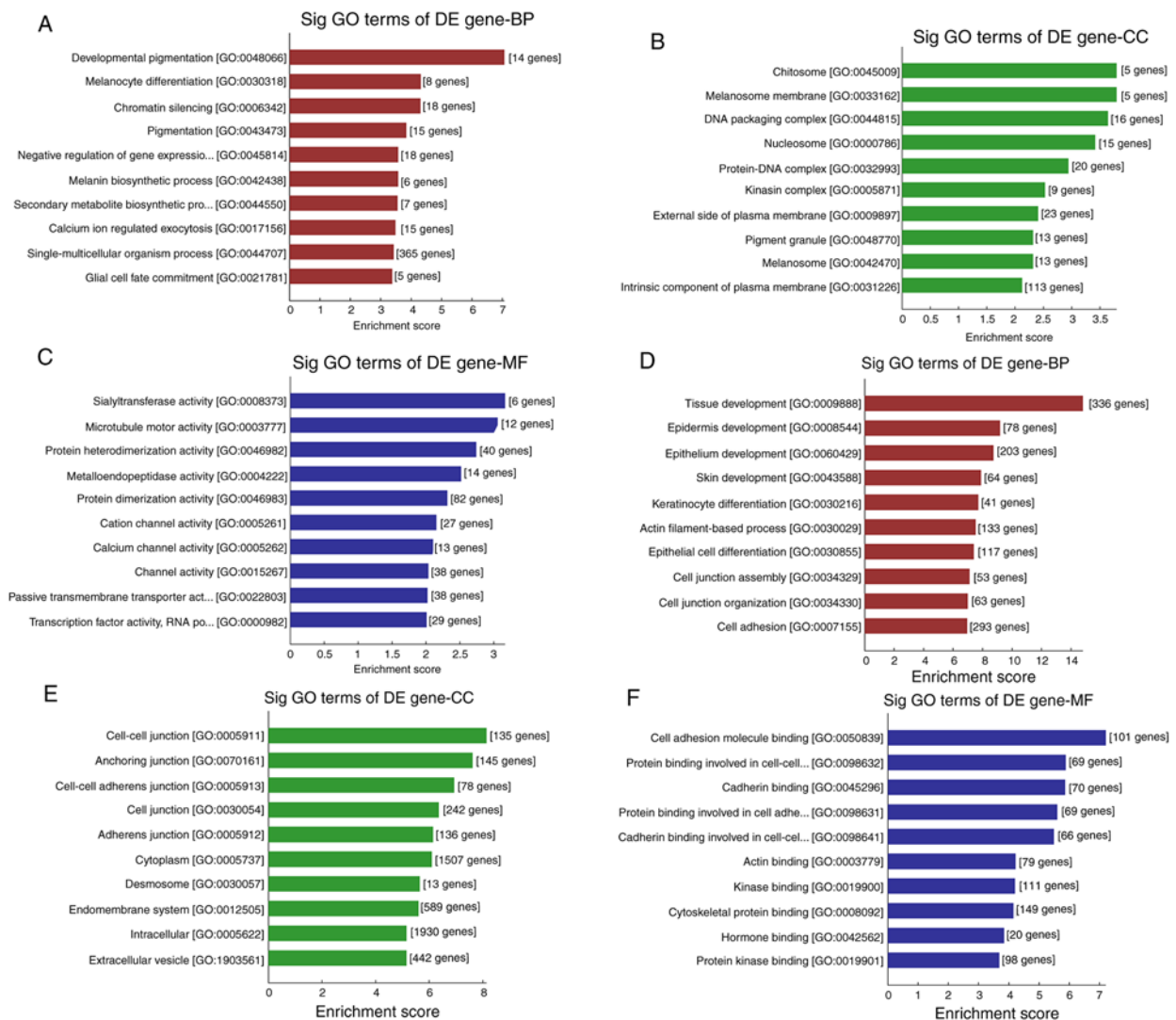


Figure 4. GO enrichment analysis. (A-F) The top 10 terms that associated with (A-C) upregulated mRNAs and (D-F) downregulated mRNAs are presented. (A and D) BP, (B and E) CC, and (C and F) MF. BP, biological process; CC, cellular component; MF, molecular function; DE, differentially expressed; GO, gene ontology.

KEGG pathway enrichment analysis results are presented in Fig. 5, including 10 pathways associated with upregulated mRNAs (Fig. 5A) and 10 pathways associated with downregulated mRNAs (Fig. 5B). These results demonstrated that the upregulated mRNAs may be involved in 'glycosphingolipid biosynthesis-ganglio series', 'transcriptional misregulation in cancer', 'toll-like receptor signaling pathway' and 'melanogenesis'. In addition, 'oxytocin signaling pathway', 'regulation of actin cytoskeleton and hippo signaling pathway', 'focal adhesion' and 'gap junction' pathways were associated with the downregulated mRNAs.

RT-qPCR validation. To confirm the previous results and detect the function of lncRNAs in ALM, five randomly selected lncRNAs (Table I) were validated by RT-qPCR, including three downregulated lncRNAs NR_004845, NR_026983 and NR_034040 (Fig. 6A-C) and two upregulated lncRNAs NR_036580 and NR_037877 (Fig. 6D and E). The results were similar to those of the microarray analysis (Fig. 6). Microarray analysis and RT-qPCR demonstrated downregulated expression of NR_004845, NR_026983 and

NR_034040 and upregulated expression of NR_036580 and NR_037877. This provided reliable confirmation of lncRNA changes determined by microarray analysis.

lncRNA and mRNA CNC network, GO enrichment and KEGG analysis. Based on the five validated lncRNAs (Table I) and their target mRNAs, the co-expression network consisting of 1,064 nodes and 1,312 connections was constructed (Fig. S1). The downregulated lncRNAs NR_004845, NR_026983 and NR_034040 correlated with 391, 141 and 150 mRNAs, respectively. The upregulated lncRNAs NR_036580 and NR_037877 correlated with 249 and 373 mRNAs, respectively.

GO and KEGG analysis of the CNC network were performed. The top 10 enriched GO terms in BP, CC and MF are presented in Fig. 7A-C. The results revealed that, in BP, target mRNAs were enriched in 'adherens junction organization', 'pigmentation' and 'adherens junction assembly'. In CC, 'intrinsic component of membrane' and 'membrane part' were the most significantly enriched terms. In addition, terms associated with channel activity and DNA binding were enriched

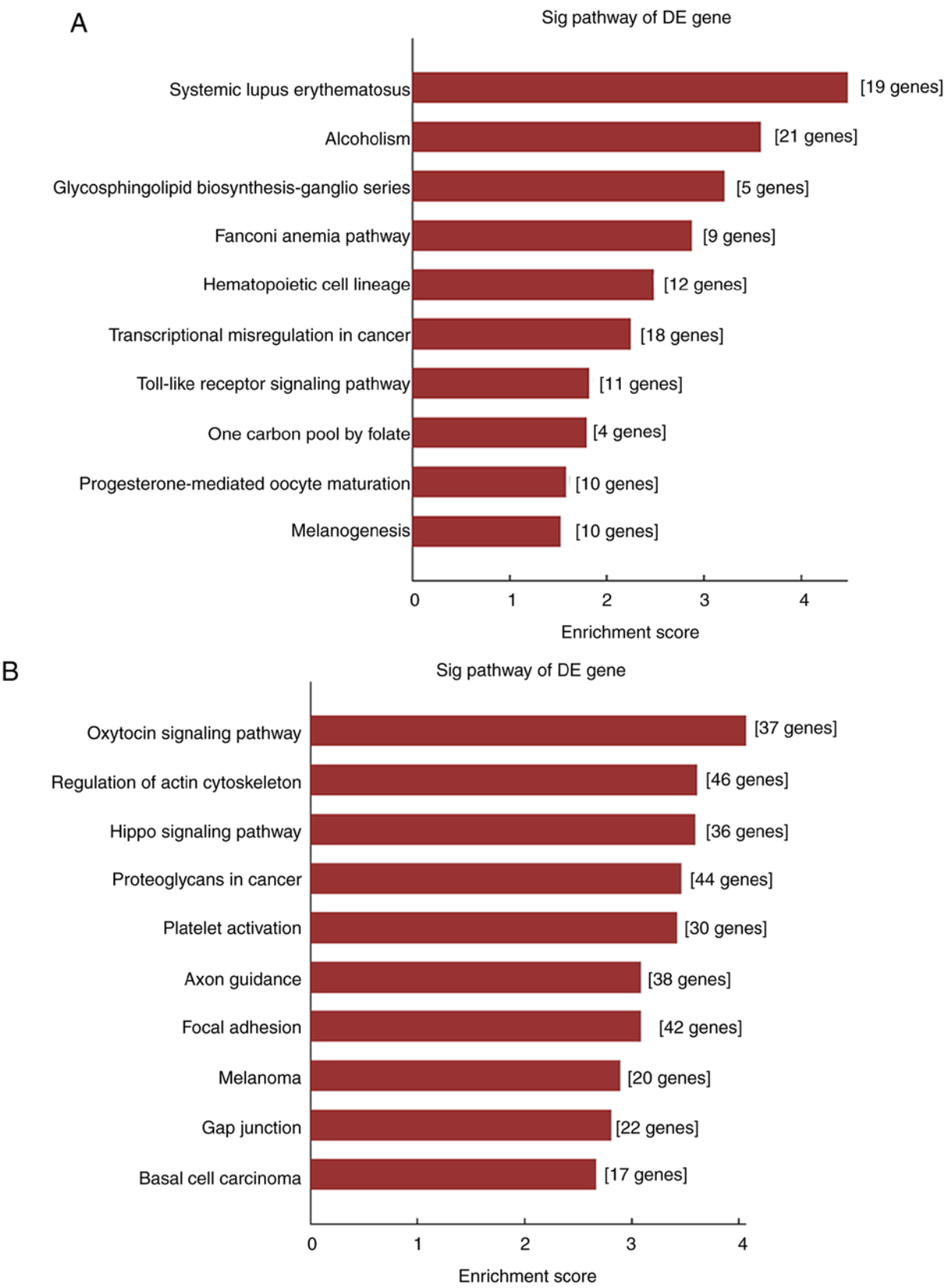


Figure 5. Kyoto Encyclopedia of Genes and Genomes pathway analysis. The top 10 pathways of (A) upregulated and (B) downregulated differentially expressed mRNAs are presented. DE, differentially expressed.

in MF. These results were in accordance with the outcome of the GO enrichment analysis of microarray results.

The KEGG analysis (Fig. 8A) demonstrated that the selected lncRNAs were associated with pathways such as

‘melanogenesis’, ‘glycosaminoglycan biosynthesis-heparan sulfate/heparin’, ‘glycolysis/gluconeogenesis’ and ‘oxytocin signaling pathway’. These results also revealed that the selected lncRNAs were representative.

Table I. Five randomly selected long noncoding RNAs for reverse transcription-quantitative PCR confirmation, construction for lncRNA and mRNA co-expression network as well as competitive endogenous RNA network construction.

Seqname	GeneSymbol	P-value	Fold-change	Regulation	Chrom	Strand	Relationship
NR_004845	LOC644936	0.000893591	8.5660466	Down	chr5	-	Intergenic
NR_026983	BTF3P11	0.002967842	8.0696294	Down	chr13	+	Intergenic
NR_034040	LGALS8-AS1	0.001504141	16.0657987	Down	chr1	-	Intronic antisense
NR_036580	DPP10-AS1	0.000199269	30.4236389	Up	chr2	-	Intronic antisense
NR_037877	LOC100505912	0.003565498	36.9781352	Up	chr4	-	Intergenic

Fold-change >2.0, P<0.05.

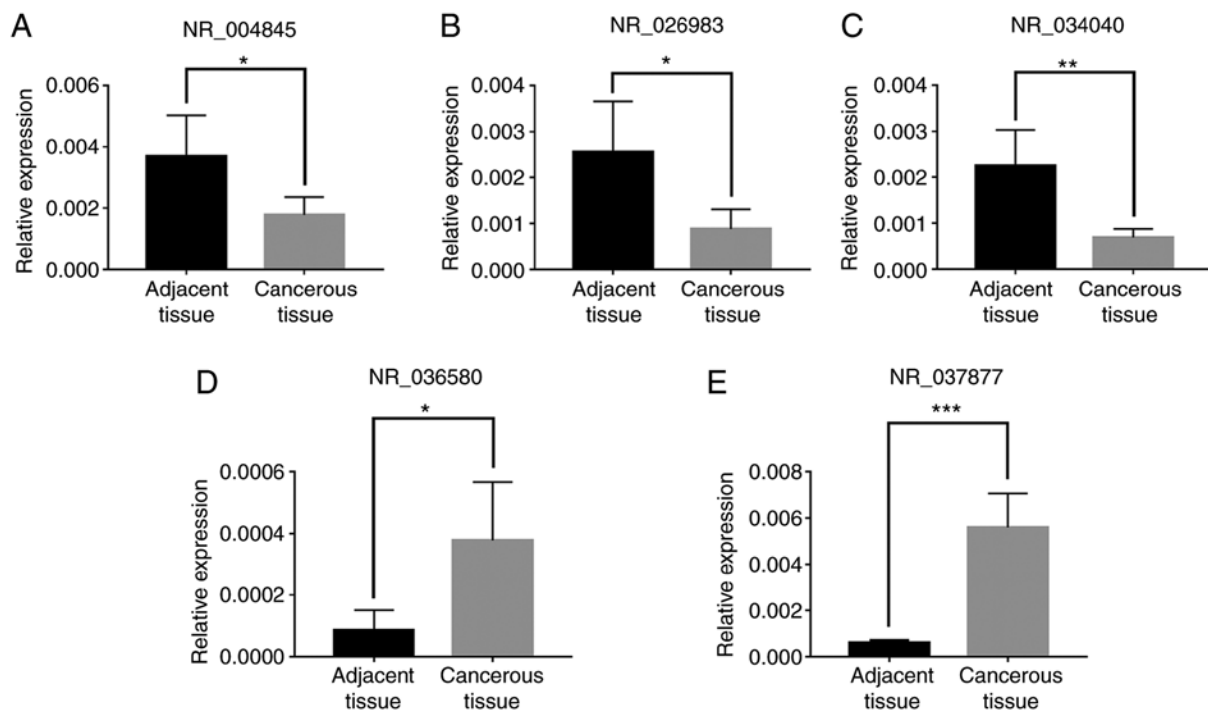


Figure 6. Validation of the microarray data by reverse transcription-quantitative PCR. The relative expression levels of differentially regulated lncRNAs are presented in ALM and adjacent non-tumor tissue: (A) NR_004845, (B) NR_026983 and (C) NR_034040 were downregulated; and (D) NR_036580 and (E) NR_037877 were upregulated in ALM. Data are presented as the mean \pm standard deviation; paired Student's t-test was used for statistical analysis. *P<0.05, **P<0.01 and ***P<0.001 vs. adjacent non-tumor tissue. lncRNA, long non-coding RNA; ALM, acral lentiginous melanoma.

Competing endogenous network analysis, GO and KEGG analysis. The theory of competing endogenous RNA (ceRNA) has revealed that a number of ceRNAs may serve a regulatory function between coding and non-coding genes via the competition of MREs. Therefore, based on the results of the microarray analysis, a ceRNA network was constructed to determine whether lncRNAs may act as ceRNAs and contribute to the occurrence of ALM. The aforementioned five randomly selected lncRNAs were used to construct this network, which is presented in Fig. S2. The light green nodes represent lncRNAs, the red nodes represent miRNAs and the light blue nodes represent mRNAs.

GO and KEGG analyses were performed to determine the potential biological action of the ceRNA network. The results of the GO enrichment analysis demonstrated that several terms associated with tissue development, such as 'developmental process', 'anatomical structure development' and 'system

development' were enriched in BP (Fig. 7D). In CC, terms such as 'cell junction' were enriched (Fig. 7E). In addition, the terms enriched in MF (Fig. 7F) were all associated with binding. These results were consistent between the microarray analysis and the ceRNA network. In KEGG analysis, pathways including 'inflammatory mediator regulation of TRP channels', 'calcium signaling pathway' and 'apelin signaling pathway' were enriched (Fig. 8B).

Discussion

No sensitive or specific biomarker for the early diagnosis and treatment of ALM is currently available. Considering the high incidence and poor outcome of ALM in Asia, especially in China, further investigations into the molecular mechanism of ALM are crucial to improve the survival rate. However, existing studies of gene mutations, epigenetics,

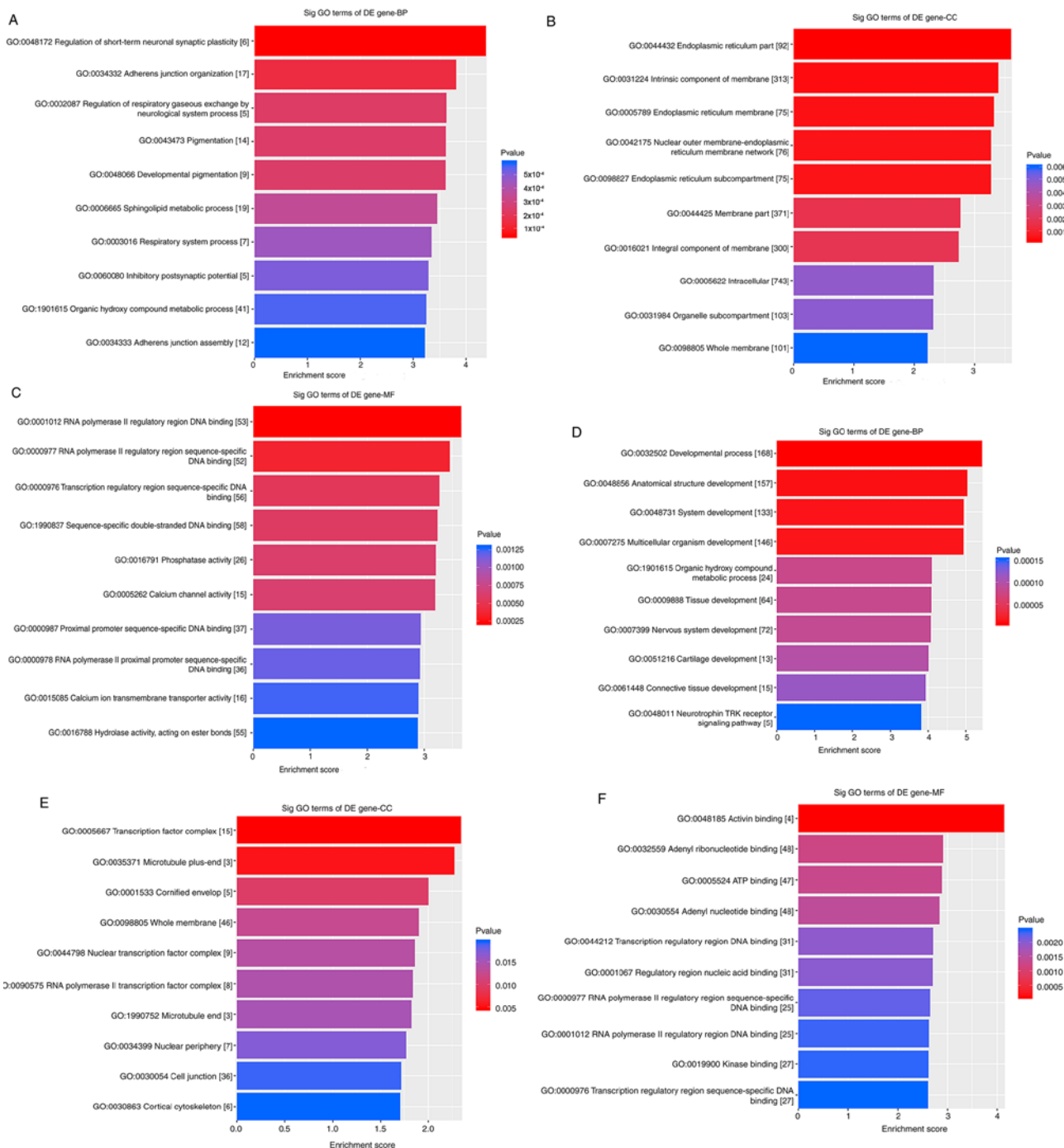


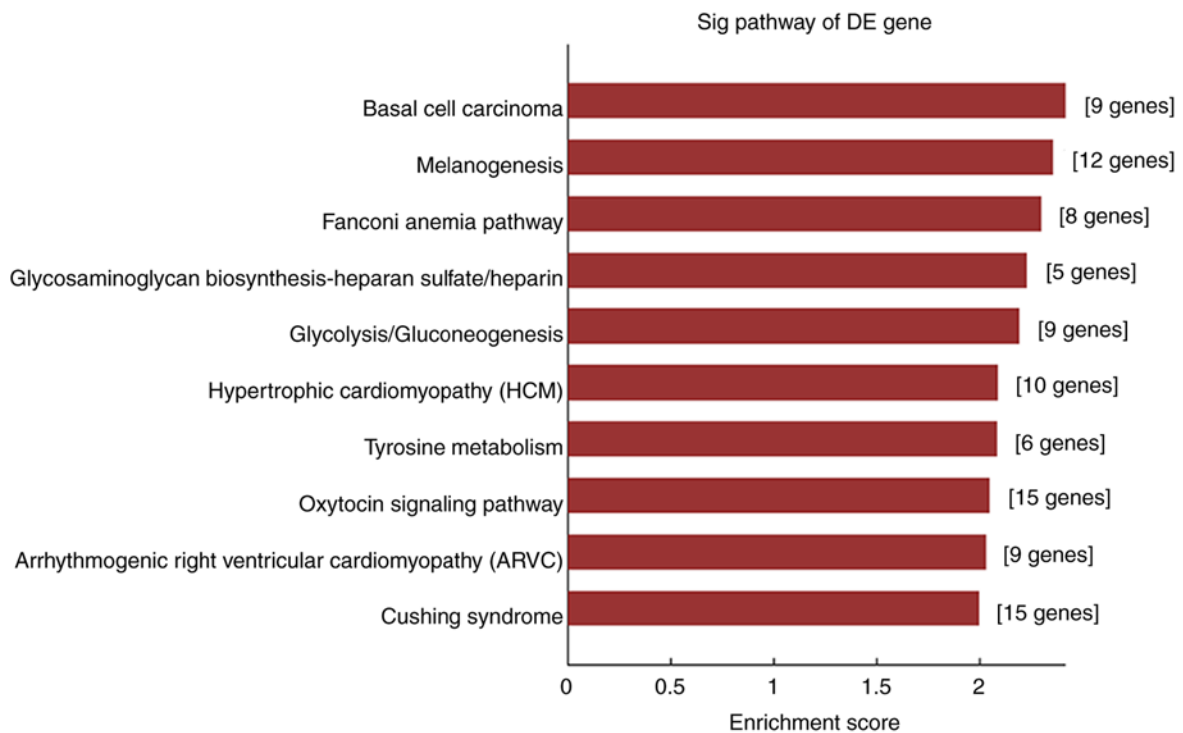
Figure 7. GO enrichment analysis in the lncRNA and mRNA co-expression network and the lncRNA/miRNA/mRNA ceRNA network. (A-C) The top 10 significantly enriched GO terms in (A) BP, (B) CC and (C) MF in the lncRNA and mRNA co-expression network. (D-F) The top 10 significantly enriched GO terms in (D) BP, (E) CC and (F) MF in the lncRNA/miRNA/mRNA ceRNA network. GO, gene ontology; lncRNA, long non-coding RNA; miRNA, microRNA; ceRNA, competing endogenous RNA; BP, biological process; CC, cellular component; MF, molecular function; DE, differentially expressed.

immune abnormalities and tumor microenvironment do not fully explain the malignant biological behaviors of ALM. The high risk of recurrence and metastasis of ALM in China has been previously attributed to patients' negligence of the disease and repeated irritation of the lesion, which is not convincing. Previously, lncRNA has emerged as a critical molecule in human cancers, such as breast and colorectal cancer, prostate, hepatocellular and basal cell carcinoma (23-26). However, a limited number of studies on lncRNAs associated with the pathogenesis, progression and

metastasis of melanoma have been published and studies on ALM are lacking.

In the present study, using the microarray analysis technology, a preliminary molecular analysis of lncRNAs and mRNAs in ALM was performed to facilitate further studies on the pathogenesis of ALM and to explore whether the biological behavior of ALM may be induced by unidentified lncRNAs. In addition, GO and KEGG pathway enrichment analyses were concluded to identify the potential functions of differentially expressed mRNAs.

A



B

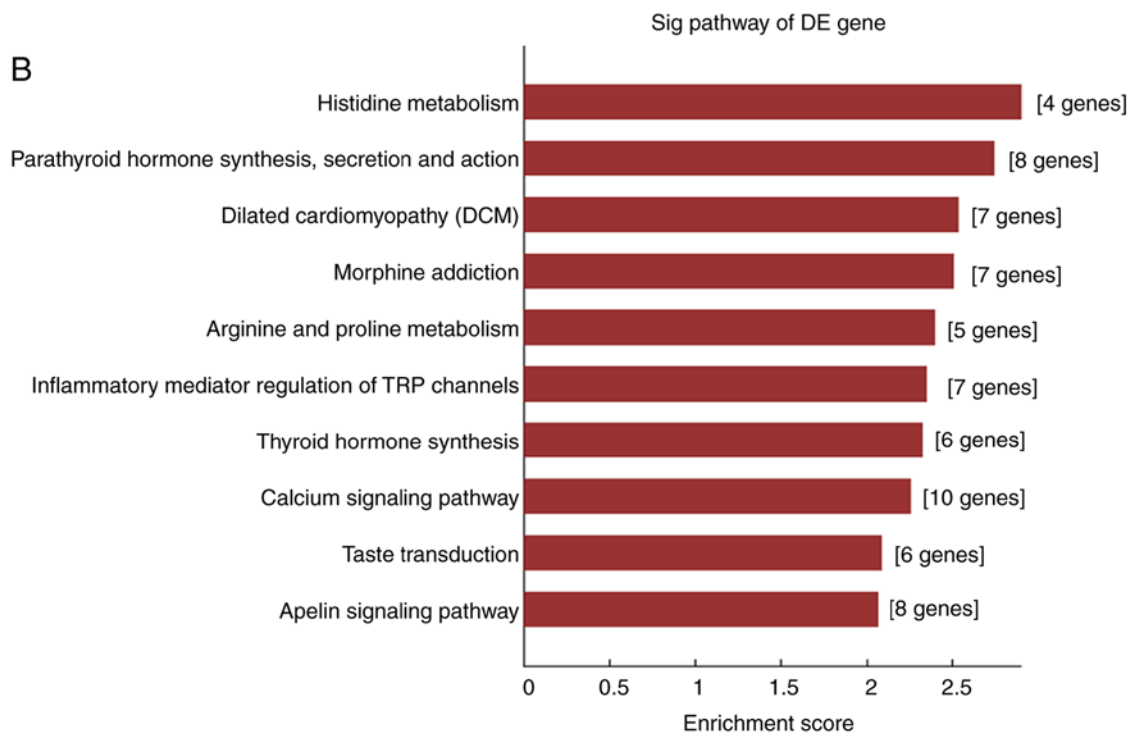


Figure 8. Kyoto Encyclopedia of Genes and Genomes pathway analysis of the lncRNA and mRNA co-expression network and the lncRNA/miRNA/mRNA ceRNA network. (A) The top 10 pathways in the lncRNA and mRNA co-expression network. (B) The top 10 pathways in the lncRNA/miRNA/mRNA ceRNA network. lncRNA, long non-coding RNA; miRNA, microRNA; ceRNA, competing endogenous RNA.

The comparison of the expression profiles of lncRNAs and mRNAs in ALM and adjacent non-tumor tissues demonstrated that 4,490 lncRNAs and 3,915 mRNAs were differentially expressed in these samples. These results were inconsistent with previous studies (8-21) of lncRNAs associated with melanoma. This may be attributed to the unique properties of ALM. The most upregulated or downregulated lncRNAs and mRNAs may help identify the molecular markers for early

diagnosis of ALM. A total of five lncRNAs were randomly selected RT-qPCR; the results were consistent with those of the microarray analysis, which suggested that the results of the microarray analysis were reliable.

GO enrichment analysis was used to identify the functions of the lncRNAs through the mRNA expression patterns. Among the upregulated mRNAs, most of the BP terms were associated with pigmentation and melanocytes. Previous

reports have demonstrated that in animal models, the inhibition of Wnt/ β -catenin signaling may lead to a decrease in melanocytes; however, inhibitors of the Wnt/ β -catenin signaling pathway do not prevent the process of pigmentation in melanoma cells (27,28). One inhibitor, ICG-001, exhibited a positive effect on pigmentation (28). Another study has demonstrated that increased pigmentation is a feature of primary melanoma with a BRAF mutation; in addition, pigmentation within the sentinel node (SN) may be associated with increased SN tumor burden and prognosis (29). The presence of pigmentation may be associated with a worse clinical outcome.

The enriched CC terms were associated with organelles that promoted the formation of melanin, such as pigment granule and melanosome. These results agree with previous studies (30,31). Compared with non-melanoma cells, melanoma cells are often characterized by different production of melanosomes. According to their morphology, melanosomes can be divided into four stages; stage IV suggests that the melanosome may be damaged (30,31). In addition to melanin synthesis, the melanosome also serves a role in clearing toxic by-products and waste during the process of melanin synthesis; this mechanism may promote the occurrence of drug resistance (30). The mediators of drug resistance may be associated with protein products such as microphthalmia-associated transcription factor, G-protein coupled receptor 143 and premelanosome protein gp100 (31). A previous study has demonstrated that silencing the expression of genes which regulate the development of the melanosome improves the sensitivity of melanoma cells to certain drugs (30). Therefore, considering the high probability of drug resistance in ALM, further studies focusing on whether the number of melanosomes in ALM is different from other types of melanoma may be useful.

In MF, channel activity-related terms were associated with upregulated mRNAs. Previous studies have demonstrated that BKCa channels regulate cell morphology and progression, as well as the migration of tumor cells (32). In addition, the expression of Na⁺ channels in tumor cells increase Na⁺-Ca²⁺ exchange, which further increases the intracellular concentration of Ca²⁺, enhancing the metastatic ability of tumor cells. Additionally, it also demonstrated that Na⁺-Ca²⁺ exchange is partly regulated by the mammalian target of rapamycin signaling pathway, which affects the proliferation and metastasis of melanoma cells (33). This is consistent with the results of the GO enrichment analysis in the present study.

For downregulated mRNAs, the BP, CC and MF terms were associated with tissue development, cell-cell junction and cell adhesion molecule binding. These terms may be associated with the progression and metastasis of ALM. Previous studies have reported that epithelial (E)-cadherin, placental (P)-cadherin and heart (H)-cadherin affect the physiological conditions of melanocytes and keratinocytes (34,35). E-cadherin and H-cadherin are often located in the basal layer of the epidermis, whereas P-cadherin is located in hair follicles. Loss of E-cadherin and expression of neural (N)-cadherin in melanoma cells are the early events in melanoma formation and metastasis (34). The expression of N-cadherin may enable melanoma cells to interact with dermal fibroblasts, which results in their migration into the dermis (35). In addition, the term 'cell adhesion' suggested that there may be numerous cell

adhesion molecules associated with melanoma that may serve as targets for inhibiting growth or invasion and improving prognosis in ALM. A previous study has demonstrated that low molecular weight heparin (LMWH) inhibited the adhesion of melanoma cells through the protein kinase C α (PKC α)/JNK signaling pathway (36). Additionally, the integrin very late antigen-4 (VLA-4), which is a crucial molecule for the invasion of melanoma cells, is inhibited by heparin. Cyr61 also serves a role in tumor formation by activating integrin-like VLA-4. A binding site for heparin has been identified in Cyr61, thus a VLA-4/Cyr61 axis may be speculated (37). This axis may be a promising target of heparin treatment in melanoma. Nitric oxide-releasing nonsteroidal anti-inflammatory drugs inhibited the function of VLA-4 and its ligand vascular cell adhesion molecule-1, thus serving as an anti-metastasis drug for melanoma (38). Hyaluronan, which is a component of the extracellular matrix, may regulate the metastasis of melanoma through cell adhesion. Overexpression of hyaluronan synthase 3 increased the amount of hyaluronan on the cell surface and induced cell cycle arrest at G1/G0, resulting in the blockage of cell adhesion and further metastasis (39). Other molecules such as activated leukocyte cell adhesion molecule, carcinoembryonic antigen-related cell adhesion molecule 1, PRL-3/PTP4A3 phosphatase and vascular endothelial growth factor may regulate cell adhesion in melanoma and its long-term prognosis (40-43). Cell adhesion detection has also been applied in the clinical diagnosis of melanoma. The expression products of genes such as β -3 integrin, cellular tumor antigen p53, laminin B1 chain and tissue-type plasminogen activator may serve a role in cell adhesion and sentinel lymph node metastasis (44). Combined detection of several of the aforementioned genes may be more effective at predicting the likelihood of nodal metastasis.

KEGG pathway enrichment analysis revealed that pathways such as 'glycosphingolipid biosynthesis-ganglio series', 'toll-like receptor (TLR) signaling pathway' and 'melanogenesis' were associated with the upregulated mRNAs; these pathways have previously been demonstrated to be involved in the pathogenesis of melanoma. 'Sialyltransferase activity' is also associated with melanoma. A previous study has demonstrated that GM3 α 2, 8-sialyltransferase (GD3 synthase) served a role in the biosynthesis of gangliosides, especially GD3 (45). In melanoma, ganglioside GD3 had been identified as a tumor-specific antigen (46). The expression of the GD3 synthase gene is activated by nuclear factor κ B (45). In addition, previous studies have reported that TLR2, 3, 4, 7, 8 and 9 are expressed on melanoma cells and may interact with the development of melanoma (47,48); TLR4 agonist lipopolysaccharide increases the proliferation of TLR4-positive melanoma cells. In addition, knockdown of TLR4 inhibited the migratory ability of melanoma cells (49). These results suggested that TLR4 signaling may contribute to melanoma progression.

Pathways such as 'oxytocin signaling pathway', 'regulation of actin cytoskeleton', 'hippo signaling pathway', 'focal adhesion' and 'gap junction' were associated with the downregulated mRNAs. Certain enhanced pathways, such as 'regulation of actin cytoskeleton', are also associated with LMWH (36). The inhibition of adhesion in melanoma cells through the PKC α /JNK signaling pathway often involves changes in the actin cytoskeleton. Another enhanced pathway

was 'hippo signaling pathway' (50-54); most of the components of this pathway are tumor-suppressor molecules. Once the pathway is activated, phosphorylated Yes-associated protein (YAP) and paralog protein TAZ accumulate in the cell plasma and induce cell cycle arrest; when these molecules are located in the nucleus, they promote cell proliferation (50). YAP and TAZ have been identified in melanoma, and the activated hippo signaling pathway may have an inhibitory effect on the development of melanoma. The expression of TAZ in invasive melanoma is higher compared with YAP, but studies speculated that patients with melanoma with high expression of YAP tend to exhibit poor prognosis (51,55,56). In addition, single nucleotide polymorphisms such as TEA domain transcription factor (TEAD) 1 and TEAD4 may also influence the survival of patients with melanoma. Therefore, these two molecules may serve as therapeutic targets for melanoma (54). A previous study has speculated that the pathogenesis of melanoma was associated with the crosstalk between hippo and mitogen associated protein kinase signaling pathways via the interaction of Raf-1 proto-oncogene serine/threonine kinase and serine/threonine kinase 3 (52).

LncRNAs affect the pathogenesis of various diseases through epigenetic regulation. To determine the exact mechanism of lncRNAs involved in ALM, a ceRNA network between lncRNAs, miRNAs and mRNAs was constructed as lncRNAs may disturb the activity of certain miRNAs, which would subsequently affect their target mRNAs. In this ceRNA network, five lncRNAs interacted with 417 mRNAs through 252 miRNAs. The results of GO and KEGG analysis were similar to those of the genes identified by microarray analysis.

Limitations existed in our study: What has been done in the present study was just a microarray analysis and its related CNC network, ceRNA network, GO analysis and KEGG analysis based on the predicted targeted genes. The further research of lncRNA function would be performed in the authors' future investigation and perhaps at that time, more evidence would be found.

In conclusion, to the best of our knowledge, the present study is the first to reveal lncRNA expression patterns in ALM using microarray analysis. The results of the present study suggested genes implicated in tissue development, pigmentation, cell adhesion activity, organelles related to melanin formation and channel activity may be involved in the pathogenesis and metastasis of ALM. In addition, the CNC and ceRNA network analysis results suggested that dysregulated lncRNAs and mRNAs may serve a role in tumor formation and development, and lncRNAs may also act as ceRNAs to disturb the pathogenesis of ALM. These molecules may be promising therapeutic targets for patients with ALM and further studies are needed to explore the precise mechanisms of ALM.

Acknowledgements

Not applicable.

Funding

The present study was supported by grants from the CAMS Innovation Fund for Medical Sciences (CIFMS-2017-12M-1-017) and the PUMC Youth Fund (grant no. 3332017168).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HS, JX, HC, YW and JS conceived this study. HS, JX, CX and WB performed the experiments and collected the data. Data were analyzed and interpreted by HC, YW and JS. HS wrote the paper. The paper was reviewed by HC, YW and JS. All authors have read and approved the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Institute of Dermatology, Chinese Academy of Medical Sciences and Peking Union Medical College (2013-LC/KY-033). All patients provided informed consents.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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