

Whole-genome identification and systematic analysis of lncRNA-mRNA co-expression profiles in patients with cutaneous basal cell carcinoma

JIAAN ZHANG*, XUYUE ZHOU*, CHENPU ZHU, YU HU, RONG LI, SHUANG JIN, DAN HUANG, MEI JU, KUN CHEN and CHAO LUAN

Institute of Dermatology, Jiangsu Key Laboratory of Molecular Biology for Skin Diseases and STIs, Chinese Academy of Medical Science and Peking Union Medical College, Nanjing, Jiangsu 210042, P.R. China

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Abstract. Cutaneous basal cell carcinoma (BCC) is a common subtype of malignant skin tumor with low invasiveness. Early diagnosis and treatment of BCC and the identification of specific biomarkers are particularly urgent. Long non-coding RNAs (lncRNAs) have been shown to be associated with the development of various tumors, including BCC. The present study conducted a comparative analysis of the differential expression of lncRNAs and mRNAs through whole-genome technology. Microarray analyses were used to identify differentially expressed (DE) lncRNAs and DE mRNAs. Reverse transcription-quantitative (RT-q) PCR confirmed the differential expression of 10 lncRNAs in BCC. Subsequently, a lncRNA-mRNA co-expression network was constructed using the top 10 DE lncRNAs. Finally, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed to investigate the possible biological effects of the identified mRNAs and to speculate on the possible biological effects of the lncRNAs. A total of 1,838 DE lncRNAs and 2,010 DE mRNAs were identified and 10 of the DE lncRNAs were confirmed by RT-qPCR. A lncRNA-mRNA co-expression network comprising 166 specific co-expressed lncRNAs and mRNAs was constructed using the top 10 DE lncRNAs. According to the results of the GO and KEGG analyses, lncRNA *XR_428612.1* may serve an important role

in mitochondrial dysfunction and the progression of BCC by modulating *TICAM1*, *USMG5*, *COX7A2*, *FBXO10*, *ATP5E* and *TIMM8B*. The present study provided whole-genome identification and a systematic analysis of lncRNA-mRNA co-expression profiles in BCC.

Introduction

Cutaneous basal cell carcinoma (BCC) is a common malignant skin tumor originating from the basal layer of the epidermis and its appendages, accounting for 65-70% of all skin tumors (1). Currently, the specific biomarkers useful for identifying BCC are still lacking and most of the studies are focused on analyses at the protein level (2-4). A small number of studies have recently reported that mRNAs may be involved in the process of BCC, but research on lncRNAs remains scarce (5-7).

Long non-coding RNA (lncRNA) is a functional RNA molecule containing >200 nucleotides, which serves an important regulatory role in gene transcription, post-transcriptional translation and epigenetic levels. Studies have suggested that lncRNAs are extensively involved in the biological process of cancer (8-12), but few lncRNA studies have clarified the roles of lncRNA in BCC. Therefore, it is a priority to explore the possible biological effects of the lncRNAs involved in BCC. Since lncRNAs have been considered regulators of mRNAs (13,14), the present study constructed a lncRNA-mRNA co-expression network for BCC with the presumption that the roles of these lncRNAs are affected by the function of related mRNAs. The lncRNA-mRNA co-expression network established bridges between lncRNAs and mRNAs to reveal the potential functional involvement of lncRNAs in BCC pathobiology. The interaction network is based on the hypothesis that once the differential expression levels of two transcripts are linearly related, a bridge can be established to demonstrate that the two are co-expressed. In this case, the two transcripts consisted of one lncRNA and one mRNA.

Overall, determining the important roles of lncRNAs in tumors is essential. Therefore, it is particularly important to conduct a whole-genome analysis of BCC tissues and construct a valid lncRNA-mRNA co-expression network, exploring the

Correspondence to: Dr Chao Luan or Dr Kun Chen, Institute of Dermatology, Jiangsu Key Laboratory of Molecular Biology for Skin Diseases and STIs, Chinese Academy of Medical Science and Peking Union Medical College, 12 Jiang Wang Miao Street, Nanjing, Jiangsu 210042, P.R. China
E-mail: luanchaopumc@126.com
E-mail: kunchen181@aliyun.com

*Contributed equally

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differentially expressed (DE) lncRNAs and making conjectures on their biological functions.

Materials and methods

Patients and samples. The study protocol was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Ethical Review Board of Peking Union Medical College (approval no. 2016-KY013). The present study recruited thirteen patients (6 male patients and 7 female patients; age range, 50-65 years) and thirteen health volunteers (6 male volunteers and 7 female volunteers; age range, 50-65 years) between February 2017 and November 2017. A total of thirteen basal cell carcinoma samples and thirteen normal tissue samples were collected from patients who underwent biopsy operation at the Institute of Dermatology, Chinese Academy of Medical Sciences. All thirteen BCC samples were pathologically confirmed and all participants provided written informed consent.

RNA extraction. Total cellular RNA was isolated from three BCC samples and three normal skin tissue samples using an RNeasy Mini kit (Qiagen GmbH) in accordance with the manufacturer's protocol and then quantified through NanoDrop ND-1000 (Thermo Fisher Scientific, Inc.). Total RNA was assessed by standard denaturing agarose gel electrophoresis.

Microarray assay. GeneChip® Human Transcriptome Array 2.0 (HTA2.0, Affymetrix; Thermo Fisher Scientific, Inc.), reportedly covers more than 245,000 coding and 40,000 non-coding RNAs of the human genome from NCBI RefSeq, UCSC, RNAdb, Ensembl, lncRNAs, UCSC, NONCODE and related literature (15). Each transcript was represented by using 1-5 probes.

The microarray hybridization was performed according to the manufacturer's standard protocols (Agilent Technologies, Inc.) including sample labeling, transcribing into double-stranded complementary (c)DNAs, cRNAs and second cycle cDNAs and then hybridizing onto the microarray. Finally, the hybridized slides were washed, fixed and scanned to images by the Affymetrix Scanner 3000 (Affymetrix; Thermo Fisher Scientific, Inc.). The data was collected by Affymetrix GeneChip Command Console (version 4.0; Affymetrix; Thermo Fisher Scientific, Inc.) and normalized by the Robust Multichip Average algorithm through the Expression Console (version 1.3.1, Affymetrix; Thermo Fisher Scientific, Inc.) software. All data were imported into Genespring software (version 12.5; Agilent Technologies, Inc.) for further analysis. The statistical significance of differentially expressed lncRNAs and mRNAs was identified via Volcano plot filtering with the threshold set of fold change ≥ 2.0 and P-value < 0.05 . Hierarchical clustering analysis was performed to reveal relationships between transcripts.

Reverse transcription-quantitative (RT-q) PCR. A total of ten BCC samples and ten normal tissues were used for RT-qPCR validation. Total RNA was isolated with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed into cDNA using the SuperScript III First Strand Synthesis System (Invitrogen; Thermo Fisher Scientific,

Inc.) according to the manufacturer's protocol. Subsequently, qPCR was performed using SYBR-Green (Takara Bio, Inc.) quantitative PCR and the ABI 7900HT Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for qPCR: 40 cycles at 95°C for 2 sec, 60°C for 20 sec and 70°C for 10 sec. β -actin was used as an internal control. The primers used for qPCR are listed in Table I. For quantitative results, the relative expression level of each lncRNA was calculated using $2^{-\Delta\Delta C_q}$ method (16) and then statistically analyzed.

Analysis of lncRNA-mRNA co-expression network. To predict the functional roles of lncRNAs, the top 10 were associated with direct regulated expression of target mRNAs using co-expression network. Pearson's correlation Coefficient (PCC) between each lncRNA-mRNA pair was calculated and those with $|PCC| > 0.99$ and $P < 0.05$ were selected. The co-expression network was visually represented using Cytoscape software (version 3.6.1; cytoscape.org), in which each mRNA corresponded to a node and two RNAs linked by an edge indicated a high correlation.

Gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) pathway analyses. GO analysis was used to identify the potential functions of significant DE genes, which covered three realms, including biological process, molecular function and cellular component (<http://www.geneontology.org>). Furthermore, the KEGG database (<http://www.genome.ad.jp/kegg/>) was conducted to harvest pathway information.

Statistical analysis. All statistical analyses were performed using the SPSS version 17.0 software (SPSS, Inc.). Unpaired Student's t-tests were performed to generate P-values. $P < 0.05$ was considered to indicate a statistically significant difference. Data are representative of three independent experiments.

Results

Microarray analysis of lncRNAs and mRNAs in BCC. To obtain the expression profiles of lncRNAs and mRNAs in BCC, three normal skin tissue samples and three BCC tissue samples were examined. The results revealed 32,904 lncRNAs and 32,552 mRNAs in total, with the results are shown in Table SI. Compared with normal samples, 524 lncRNAs (red dots in Fig. 1A) and 1,207 mRNAs (red dots in Fig. 1B) were upregulated ($P < 0.05$; fold change > 2) in BCC. In contrast, 1,314 lncRNAs (green dots in Fig. 1A) and 803 mRNAs (green dots in Fig. 1B) were significantly downregulated ($P < 0.05$; fold change > 2). The top 200 differentially expressed lncRNAs and mRNAs are presented in the hierarchical cluster analysis heat map (Fig. 2A and B), in which 'Normal' represents three normal skin tissue samples and 'BCC' represents three BCC tissue samples.

RT-qPCR validation of differentially expressed lncRNAs. The top 10 DE lncRNAs were selected for RT-qPCR analysis to verify the microarray results in ten BCC tissue samples and ten normal tissue samples. The RT-qPCR results demonstrated that four lncRNAs (*XR_428611.1*, *XR_428612.1*, *ENST00000566225.1* and *ENST00000430816.1*) were downregulated in BCC and six lncRNAs (*NR_002160.1*,

Table I. Primers used for reverse transcription-quantitative PCR.

lncRNA	Forward primer (5'→3')	Reverse primer (5'→3')
<i>XR_428611.1</i>	GGCTCGCTCTCCACTCCATCC	TTACACACCCCTTCTCCCTTCCAG
<i>XR_428612.1</i>	TGCTGCTGGCGAGAAGAATGC	ATGTTGAAGACCGGGGCTTTCTG
<i>ENST00000566225.1</i>	GGAAGGGCACCACCTTCTACC	AATTACCTGTGAAGGCCCCG
<i>ENST00000430816.1</i>	CAGCCAACCACGCAGACCTG	TTCCAGAACAGCACGAAGTCCAC
<i>NR_002160.1</i>	TGACCGTCAACTTGGGATTGT	TTAAACAAGCCAGCCAAGCG
<i>ENST00000444265.1</i>	CTGCCAAGAGGAATCCAGCA	TCTGGGTTTTCCATGCGTGT
<i>HIT000067334</i>	TGCATGTAAGTGGCAAAGATGA	TGCTTCTCCAGAATGGCTTGA
<i>ENST00000598252.1</i>	GGAGGCAGATTCAGTCAGACG	TGGACAAAAACTCCAGTGTGC
<i>uc003hcu.1</i>	GTTTTCCCCCTCACCAAGCA	GCGACCAAAGCCACAATAGC
<i>ENST00000548135.1</i>	TGCCCAGGAAGCTGAGGTGAG	AGCTGCTGGAAGGCGAGGAG
<i>β-actin</i>	TCCTTCCTGGGCATGGAGT	AGCACTGTGTTGGCGTACAG

lncRNA, long non-coding RNA.

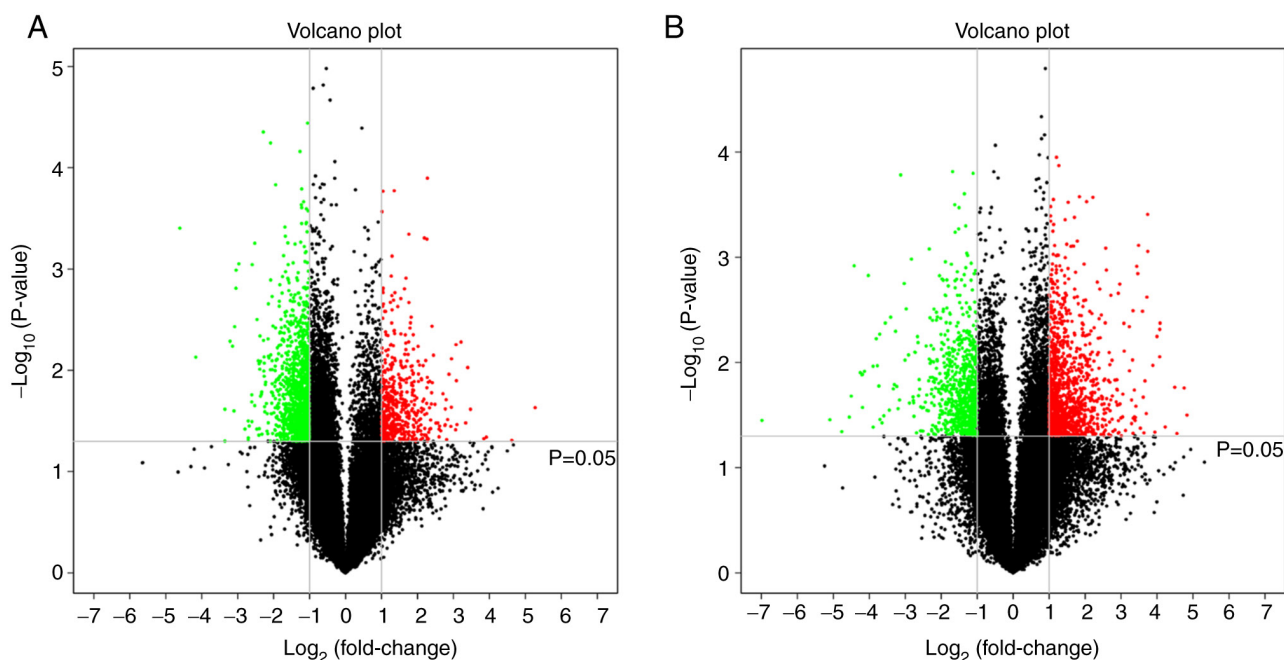


Figure 1. DE lncRNAs and DE mRNAs. The expression of lncRNAs and mRNAs were compared between BCC and normal cutaneous tissue specimens. Volcano plot of lncRNAs (A) and mRNAs (B) illustrate the difference in the expression levels of each transcript. The red dots represent transcripts significantly upregulated in BCC and the green dots represent transcripts significantly downregulated in BCC, based on the statistical criteria (fold change >2; P<0.05). The horizontal axis represents fold of change in expression (on a \log_2 scale) and the vertical axis represents P-value (on a negative \log_{10} scale). In the plot, the vertical line on the left stands for a two-fold downregulation while the right one stands for a two-fold upregulation and the horizontal line represents P=0.05. DE, differentially expressed; lncRNA, long non-coding RNA; BCC, cutaneous basal cell carcinoma.

ENST00000444265.1, *HIT000067334*, *ENST00000598252.1* and *uc003hcu.1*, *ENST00000548135.1*) were upregulated in BCC. As shown in Fig. 3, the relative values of the expression levels detected by RT-qPCR were found to be consistent with the microarray data. This result suggests that the transcript identification and abundance estimates were highly reliable.

Construction of a lncRNA-mRNA co-expression network. To construct the lncRNA-mRNA co-expression network in BCC, the correlation of the expression levels of the top 10 lncRNAs and individual mRNAs in the samples were investigated by

Pearson correlation coefficient (PCC) and tested the reliability of PCC by P-value. A strict positive correlation and a negative correlation is indicated by a PCC equal to 1 or -1 (17). The present study limited the PCC value to >0.99 or <-0.99 and set the P-value to <0.05, which is a strict standard used to establish a correlation (18).

The lncRNA-mRNA network we constructed consisted of 10 lncRNA nodes (green rectangles in Fig. 4), 150 mRNA nodes (purple dots in Fig. 4) and 166 edges. Different edge types represent different interrelationships. As shown in the figure, *ENST00000598252.1* is co-expressed with 54 of the 150 mRNAs

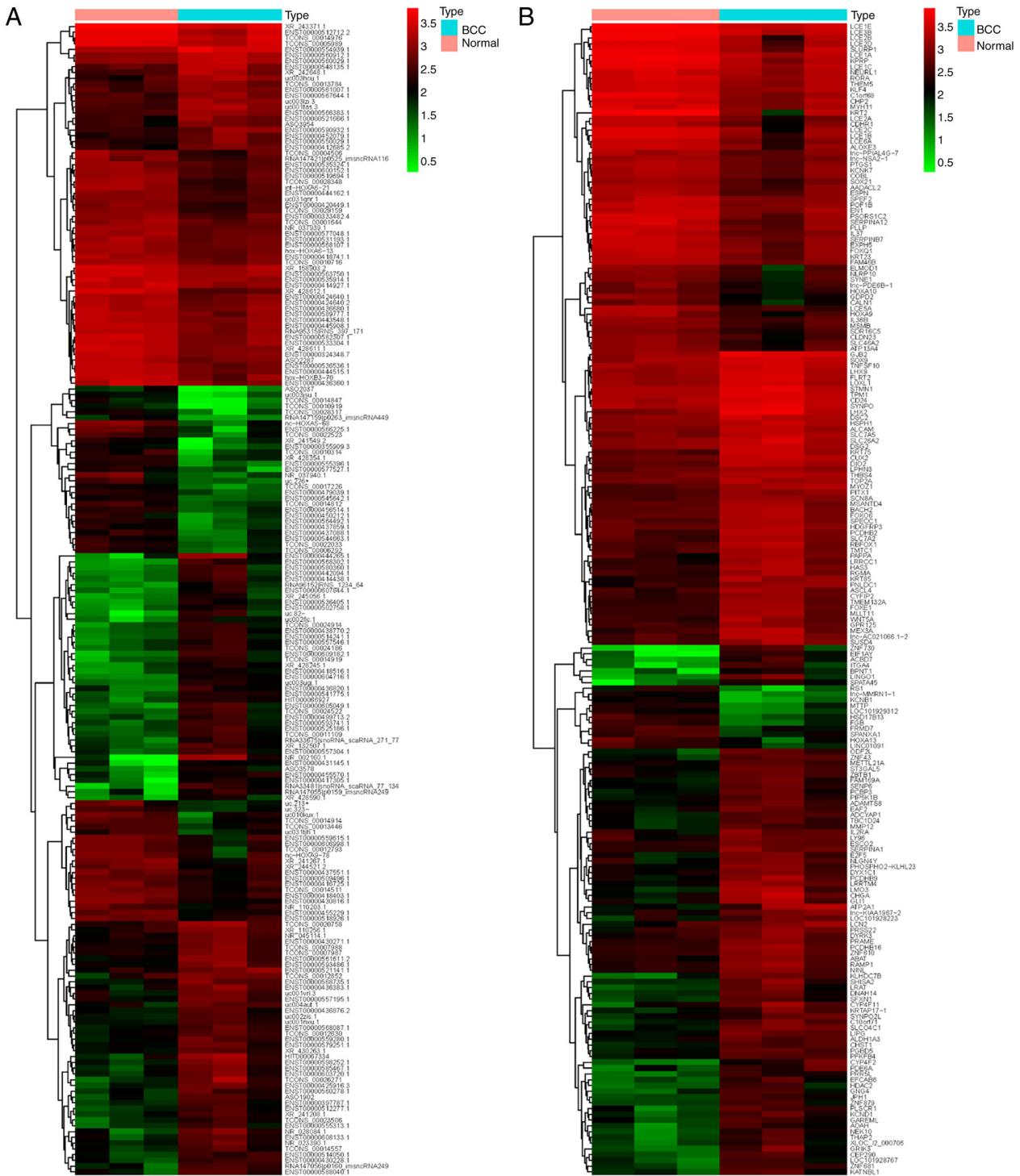


Figure 2. Hierarchical Clustering analysis of DE lncRNAs and DE mRNAs. Hierarchical Clustering analysis of (A) lncRNAs and (B) mRNAs depict the top 200 difference in the expression levels of each transcript within two groups (normal cutaneous tissue samples and BCC samples). Red, high relative expression. Green, low relative expression. DE, differentially expressed; lncRNA, long non-coding RNA; BCC, cutaneous basal cell carcinoma tissue.

identified, the most of all identified DE lncRNAs. The next most abundant, *NR_002160.1*, was co-expressed with as many as 38 mRNAs. Moreover, *TICAM1* mRNA was co-expressed with the maximum number (three) among the 10 DE lncRNAs.

GO enrichment and pathway analysis. To further explore potential targets of the DE lncRNAs in BCC progression, GO enrichment and KEGG pathway analyses were performed on the DE mRNAs (Fig. 5).

Numerous GO terms were targeted by these coding mRNAs, including 3,625 biological processes, 635 molecular functions and 446 cellular components. The target genes and detailed information are represented in Table SII. GO analysis is widely used to identify a set of gene products in three categories: Biological processes, cellular components and molecular functions (19). For the DE mRNAs in BCC identified by microarray, the most significant emerging themes as determined by P-value were ‘keratinization’ (biological process; Fig. 5A blue),

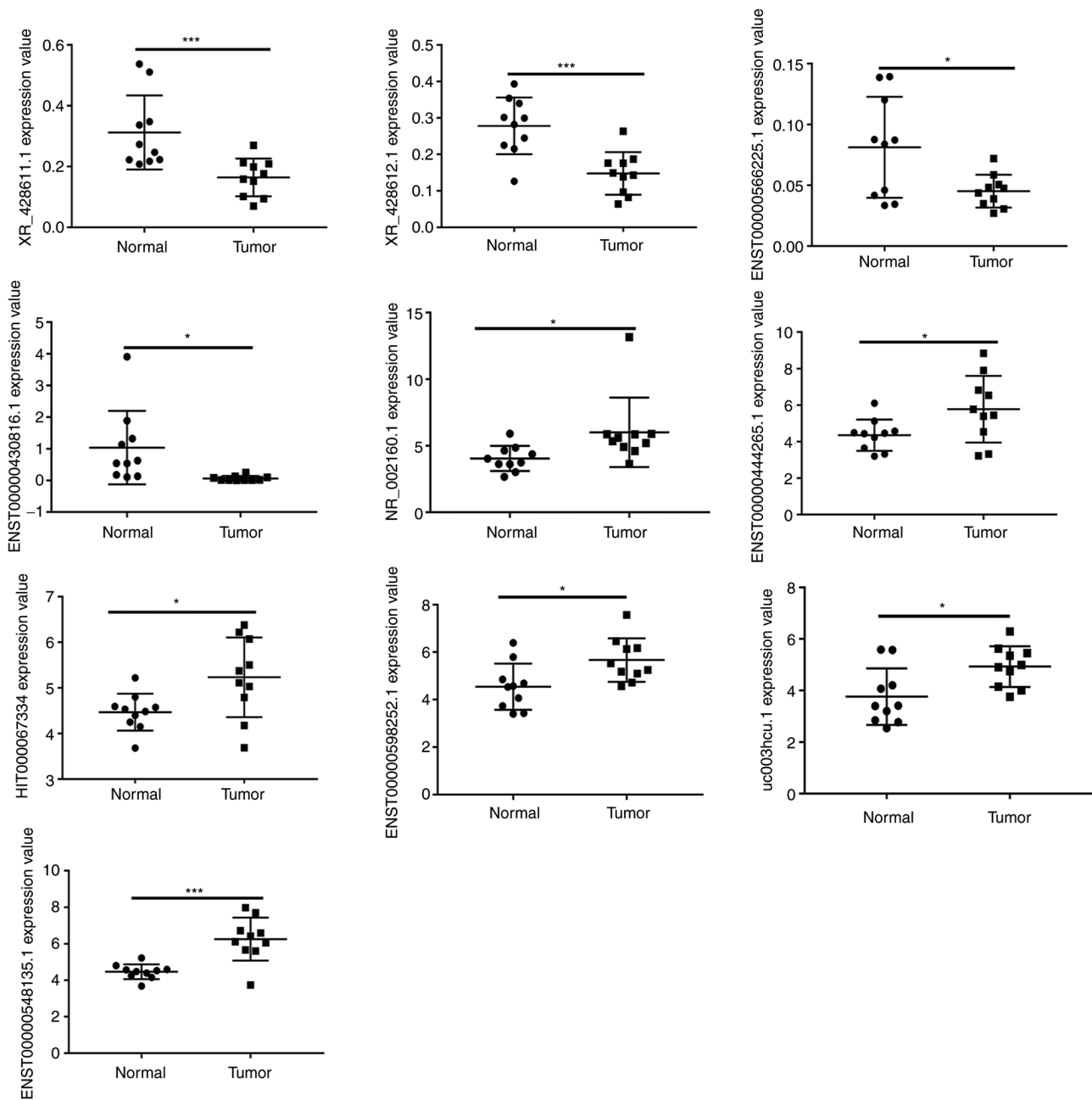


Figure 3. Validation for the DE lncRNAs by RT-qPCR. The expression levels of ten lncRNAs were compared between cutaneous basal cell carcinoma and normal skin tissues. The vertical axis represents the expression value computed from the qPCR data, which are consistent with the data from microarray. * $P < 0.05$ and *** $P < 0.001$. DE, differentially expressed; lncRNA, long non-coding RNA; RT-qPCR, reverse transcription-quantitative PCR.

'cornified envelope' (cellular component; Fig. 5A yellow) and 'unfolded protein binding' (molecular function; Fig. 5A red).

In addition, 152 KEGG pathways enriched with the DE mRNAs were detected. For KEGG pathway analysis, the top 20 of the 152 identified KEGG pathways were selected and analyzed. The majority of the KEGG pathways most enriched included the p53 signaling pathway, fructose and mannose metabolism, protein processing in the endoplasmic reticulum, microRNAs in cancer and apoptosis (Fig. 5B).

Discussion

The role of lncRNAs in cancer has been recognized and a number of studies have been conducted with lung cancer,

breast cancer, liver cancer, gastrointestinal tumors and skin cancer (20-22). lncRNAs can be upregulated or downregulated in a variety of cancer processes, including cutaneous tumors, affecting upstream and downstream target genes, signaling pathways and metabolic pathways and ultimately promoting tumor proliferation and invasion (23). As a common cutaneous tumor, BCC has also been studied to determine its possible pathogenesis. It is currently hypothesized that abnormal activation of the Hedgehog pathway induced by the *PTCH1* gene serves a major role in BCC (24). In addition, abnormal upregulation of the *SMO* gene, loss of tumor suppressor p53 and activation of the Hippo-Yes-associated protein pathway are considered to be involved in the progression of BCC. Some oncogenes, such as *ARID1A*, *CASP8*, *CSMD1*, *GRIN2A*,

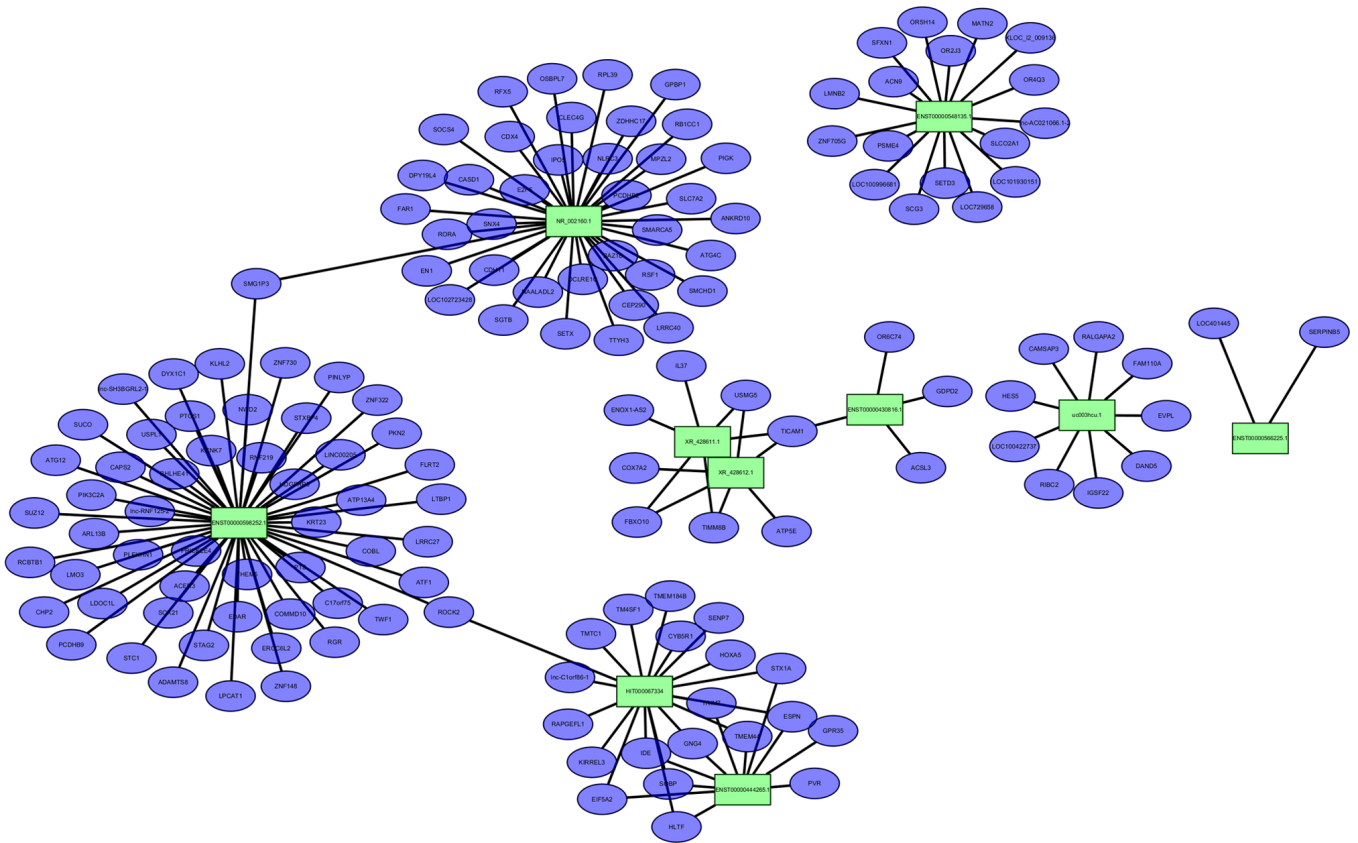


Figure 4. lncRNA and mRNA co-expression networks in cutaneous basal cell carcinoma tissues. The co-expression network consists of the top 10 DE lncRNAs (green rectangles), correlated 150 mRNAs (purple dots) and 166 edges. Each edge represents correlation r value >0.99 or <-0.99 with $P < 0.05$. lncRNA, long non-coding RNA; DE, differentially expressed.

KRAS, *NOTCH1*, *NOTCH2*, *NRAS*, *PIK3CA*, *PREX2* and *RAC1*, are also mutated in BCC (25,26). In summary, lncRNAs are currently important for cancer study, but their role in BCC is seldom mentioned and remains unclear.

To explore the potential role of lncRNAs in the processes of BCC, the present study performed whole-genome identification of lncRNAs using an array to detect the expression profiles of lncRNAs and mRNAs in three normal tissue samples and three BCC tissue samples. A total of 32,904 lncRNA probes and 32,552 mRNA probes were detected and a number of RNAs were found to be differentially expressed, including 1,838 lncRNAs and 2,010 mRNAs. The selection criteria, fold change >2 and $P < 0.05$, ensured the significance of the differential expression data. The top 10 DE lncRNAs, including four downregulated lncRNAs and six upregulated lncRNAs, were verified through RT-qPCR. The RT-qPCR results showed a trend in expression levels consistent with those of the lncRNA array analysis.

Some of the DE lncRNAs identified in the present study have been reported to be involved in carcinogenesis; for example, the p27963 probe detected lncRNA *nc-HOXA5-68*, one of the top 10 downregulated DE lncRNAs (27). As suggested by its name it has a regulatory effect on the proto-oncogene *HOXA5*. *HOXA5* can affect various systemic systems and can serve a pivotal role in the cancer process (28). The first studied lncRNA *HOTAIR* can also be regulated to induce cancer progression, such as liver cancer, cervical cancer and cutaneous melanoma (29,30).

Although lncRNAs do not encode proteins directly, they can be involved in various biological processes including the progression of cancer by interacting with other biomolecules such as transcription factors, miRNAs, mRNAs and RNA-binding proteins to form gene regulatory networks (31). Several studies have been conducted to obtain lncRNA biomarkers associated with tumor development and prognosis by constructing lncRNA-mRNA networks, such as thyroid cancer, hepatocellular carcinoma and ovarian cancer (32-34). To further explore the potential role and mechanism of lncRNAs in BCC, the present study constructed a lncRNA-mRNA co-expression network focusing on the top 10 specific DE lncRNAs. The most striking example involved the largest downregulation of lncRNA, *XR_428612.1*, in BCC. This lncRNA has not been studied, but the co-expression network demonstrated that it has a co-expression relationship with six mRNAs, *TICAM1*, *USMG5*, *COX7A2*, *FBXO10*, *ATP5E* and *TIMM8B*. Notably, all these mRNAs are reported to be associated with mitochondrial dysfunction and glucose metabolism (35-41). In the co-expression network of the present study, lncRNA *XR_428612.1* was positively correlated with TIR domain-containing adaptor molecule-1 (*TICAM1*). It has been reported that *TICAM1* downregulation is an essential step in Toll-like receptor 3 (TLR3) activation and stops TLR3-mediated IFN production (35). Activated TLR3 induces the upregulation of transactivating p63 isoform α (TAp63 α), a p53-related protein that downregulates the expression of Bcl-2, an anti-apoptosis molecule and induces cell apoptosis in a caspase-dependent manner through death receptors and mitochondria (36). The



Figure 5. GO and KEGG pathway analysis of DE mRNAs in BCC. (A) The 20 most enriched GO terms (vertical axis) for DE mRNAs in BCC selected based on P-value (horizontal axis). The enriched GO terms at the vertical axis are grouped into 'Biological Process' (blue), 'Cellular Component' (yellow) and 'Molecular Function' (red). (B) The 20 most enriched KEGG pathways (vertical axis) for DE mRNAs in BCC are selected based on P-value (horizontal axis). The most enriched KEGG pathway is at the top of the vertical axis. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DE, differentially expressed BCC, cutaneous basal cell carcinoma tissue.

present study noted that *TICAM1* was co-expressed with the maximum number (three) of lncRNAs, which indicated that it may serve an important role in the pathogenesis of BCC. In addition, *FBXO10* is also thought to control the degradation of Bcl-2 and to regulate cell apoptosis (37). TIMM8B, a mitochondrial ribosomal protein (MRP), has been reported to be upregulated in colon mucosa carcinogenesis mediated by diabetes (28). MRPs may be affected by glucose metabolism and participate in proliferation, metastasis, or cellular migration in malignancies (38). In addition, diabetes-associated protein in insulin-sensitive tissues (DAPIT) encoded by *USMG5* (39), ATP synthase epsilon subunit (ATP5E) and cytochrome c oxidase subunit 7A2 (COX7A2) are partial components of ATP synthase and participate in the interconversion of mitochondrial oxidative phosphorylation (OXPHOS) and glycolysis, both of which are sources of ATP (40,41). The upregulation of these transcripts clearly contribute to modulating tumor anabolism, redox and calcium homeostasis, apoptosis and metastasis by governing mitochondrial function.

In the present study, GO and KEGG pathway analyses showed significant changes among the DE mRNAs. KEGG analysis found that the 'p53 signaling pathway', 'fructose and mannose metabolism', 'protein processing in the endoplasmic reticulum', 'microRNAs in cancer' and 'apoptosis' were the top five significantly changed pathways. P53 has been reported to be overexpressed in BCC (42). It interferes with Bcl-2 in mitochondria, which results in the release of cytochrome c and promotes apoptosis by activating the mitochondrial pathway (42,43). In fact, three of the five top significant KEGG pathways, including the 'p53 signaling pathway', 'fructose and mannose metabolism' and 'apoptosis', were aligned with the carcinogenic mechanisms of the mRNAs co-expressed with lncRNA *XR_428612.1*, as aforementioned. Since the construction of lncRNA-mRNA co-expression network and KEGG analysis relationships are independent, this outcome greatly increases the reliability of the present study. Therefore, it was hypothesized that mitochondrial dysfunction involving OXPHOS and glycolysis modulated by lncRNA *XR_428612.1* may serve a considerable role in the progression of BCC and deserves to be the focused of a follow-up study.

In conclusion, the present study provided a comprehensive analysis of lncRNA-mRNA co-expression profiles of patients with cutaneous basal cell carcinoma. GO and KEGG analyses further revealed the function of mRNAs and suggested the possible biological effects of lncRNAs. The results indicated that lncRNA *XR_428612.1* may serve an important role in mitochondrial dysfunction and the progression of BCC by modulating *TICAM1*, *USMG5*, *COX7A2*, *FBXO10*, *ATP5E* and *TIMM8B*. These data argue for further intensive research to provide a more comprehensive understanding of the molecular mechanisms underlying the metabolism of BCC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JZ, XZ, DH, MJ, CL and KC conceived and designed the study. DH, YH, RL and SJ collected the samples and acquired the data. JZ, XZ and CZ interpreted or analyzed data. XZ, CZ, YH, RL and SJ prepared the manuscript, which was revised for important intellectual content by JZ, CL and KC. All authors reviewed and approved the final manuscript. CL and KC confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The study protocol was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Ethical Review Board of Peking Union Medical College (approval no. 2016-KY013). All participants provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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