Original Article

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Association of biobehavioral factors with non-coding RNAs in cervical cancer

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Summary **In order to elucidate the mechanisms underlying the biobehavioral factors responsible for cervical cancer from the perspective of lncRNAs. Tumor samples were obtained from patients with stage Ib-IIb squamous cervical cancer, which were divided into high- and low-risk groups according to biobehavioral risk factors. A lncRNA + mRNA microarray was performed, and the results were validated using qRT-PCR. Gene ontology (GO), pathway, and lncRNA-mRNA co-expression analysis were performed to predict the potential functions of the differentially expressed transcripts. 1,621 lncRNAs and 1,345 mRNAs were found to be differentially expressed between the high-risk and low-risk groups. The results of the qRT-PCR validation were in 100% agreement with the microarray analysis results. GO analysis revealed that the transcripts showing significantly different expression were mainly associated with various aspects of immune response. Pathway analysis indicated that systemic lupus erythematosus signaling was the most significantly down-regulated pathway in the high-risk group. Co-expression analysis indicated NONHSAT002712, NONHSAT095060, and TCONS_00026535 had significant correlations with ZNF683 and BTLA, which were found to be associated with the GO term "adaptive immune response". The levels of genome-wide lncRNAs are significantly altered in cervical tumors from patients with higher biobehavioral risk factors.**

Keywords: Cervical cancer, biobehavioral factor, psychoimmunology, adaptive immune response

1. Introduction

Cervical cancer is the second most commonly diagnosed cancer and the third leading cause of cancer death among females in developing countries. Among the females diagnosed with cervical cancer, many are diagnosed at the advanced stages of the disease where they have limited treatment options and show poor prognosis (*1*). There were an estimated 98,900 new cervical cancer cases and 30,500 related deaths in China in 2015 (*2*).

Cervical cancer patients experience significantly more depression and anxiety than the general population (*3*). Women who receive results of an abnormal Pap smear or a positive HPV DNA test may often experience unwarranted fear, distress, and anxiety about cervical cancer (*4*). A recent analysis indicates that 25.7% of early stage cervical cancer (ECC) cases and 22.2% of locally advanced cervical cancer (LACC) cases have elevated anxiety levels (*5*).

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In addition, cervical cancer survivors are more likely to have a lower quality of life (QOL) and higher levels of depression and anxiety (26% and 28%, respectively) compared to other cancer survivors (*6*).

Biobehavioral factors such as depression, anxiety, and stressful life events have long been suspected to underlie cancer progression (*7*). Animal studies have also revealed that behavioral stress can promote the progression of ovarian cancer, breast cancer, pancreatic cancer, and several other types of malignant tumors (*8,9*). The mechanisms underlying the effects of biobehavioral stress on cancer progression have been studied through psychoneuroimmunology (*7,10*), and these studies have shown that biobehavioral factors influence the neuroendocrine regulation of hormones (*i.e.*, catecholamine neurotransmitters, dopamine, adrenaline, and noradrenaline), which may impair the immune response and contribute to cancer onset and development.

Long noncoding RNAs (lncRNAs) are transcripts longer than 200 nucleotides with no apparent protein-coding role. LncRNAs are involved in numerous important biological processes such as chromatin modification, genomic imprinting, and enzymatic activity regulation (*11*). The overexpression, deficiency, or mutation of lncRNAs has been implicated in various malignant tumors and other human diseases (*12*). However, few studies have evaluated the changes and potential functions of lncRNAs in cervical cancer patients experiencing higher psychological stress.

In the current study, we performed a high-throughput analysis to compare the lncRNA and mRNA expression profiles between high- and low-psychological stress cervical cancer groups. Our aim was to discover the mechanisms underlying the potential psychoimmunological effects of behavioral stress on cervical cancer from the perspective of lncRNAs. Our findings provide new insights into the psychological epidemiology of cervical cancer.

2. Materials and Methods

2.1. *Patients*

Women older than 18 years of age with an abnormal Pap test result suspected for cervical carcinoma were determined to be potentially eligible for this study. The study inclusion was confirmed after the histologic diagnosis of squamous cell carcinoma of the uterine cervix. Patients with a previous cancer diagnosis, regular use of a systemic steroidal medication in the last 4 months, comorbidities known to alter the immune response (such as autoimmune diseases), or the inability to accurately answer questions were excluded. This study was approved by the Ethics Committee of the Obstetrics and Gynecology Hospital affiliated to Fudan University.

2.2. *Psychological measures*

2.2.1. *Depression*

Depressive symptoms were assessed using the Center for Epidemiologic Studies Depression Scale (CESD) and the Zung's Self-rating Depression Scale (SDS). The CESD is a validated 20-item scale that assesses depressive symptoms occurring in the prior week. Scores of 16 or higher indicate a high biobehavioral risk (*13*). Four subscales of CESD have been confirmed by factor analysis: depressed affect, vegetative depression, positive affect, and interpersonal relationships. On the other hand, the SDS is a 20-item self-reported scale that assesses psychological and somatic symptoms of depression. Cut-off scores for the SDS were as follows: < 50 = normal, 50 to 59 = mild depression, 60 to 69 = moderate depression, and > 69 = severe depression (*14*).

2.2.2. *Social support*

Social support was assessed by the Chinese version of the Social Support Rating Scale (SSRS), which demonstrates good validity and reliability among Chinese populations (*15*). The SSRS consists of ten items that measure three dimensions of social support: objective support, subjective support, and support utilization.

2.2.3. *Anxiety*

The Zung's Self-rating Anxiety Scale (SAS) was used to quantify the level of anxiety (*16*). The self-reported questionnaire contained 20 items. Cut-off scores were as follows: < 45 = normal, 45 to 59 = mild to moderate anxiety, 60 to 74 = marked to severe anxiety, and > 75 = extreme anxiety.

2.2.4. *Sleep quality*

The Pittsburgh Sleep Quality Index (PSQI) is a 19-item self-reported questionnaire used to assess the type and frequency of sleep disturbances occurring in the prior month (*17*). A global score greater than 5 indicated poor sleep.

2.3. *Tissue collection and RNA extraction*

Six cervical carcinoma samples were harvested during the laparoscopic surgery from each study participant. All the samples were rapidly frozen in liquid nitrogen, followed by storage at -80°C. Total RNA was extracted from the frozen tumor tissues using the mirVana RNA Isolation Kit (Ambion). RNA concentration and purity were then determined using the NanoDrop ND-2000 Spectrophotometer (Thermo Fisher Scientific). RNA integrity was determined by standard denaturing

agarose gel electrophoresis.

2.4. *Microarray analysis*

Total RNA was labeled using the Quick-Amp Labeling Kit, One-Color (Agilent Technologies) and hybridized onto the Agilent Human lncRNA Array (4*180K). The chip detected 46506 human lncRNAs and 30656 human mRNAs. Hybridization signals were detected using the Microarray Scanner (Agilent p/n G2505C). Agilent Feature Extraction Software was utilized to extract the raw data. Quantile normalization and subsequent processing of the data were carried out using GeneSpring Software version 12.0 (Agilent Technologies). Differentially expressed genes were defined as those with an absolute value of fold change (FC) > 2 and a p-value < 0.05 (Student's t-test). Microarray profiling was performed by OE Biotech, Shanghai, China.

2.5. *Quantitative real-time PCR assay*

Total RNA was reverse transcribed into cDNA using the PrimerScript RT Kit with gDNA Eraser (Takara, Shiga, Japan) according to the manufacturer's standard protocols. Quantitative real-time PCR (qRT-PCR) was performed on an Applied Biosystems Vii^{TM} A7 System (Life Technologies, Tokyo, Japan). Each 20-μL reaction contained 10 μL of SYBR Premix Ex Taq II (2×) (Takara), 2 μL of cDNA, 0.8 μL of the forward primer, 0.8 μL of the reverse primer, and 6 μL of dH_2O. The PCR cycling conditions were as follows: incubation at 95°C for 10 min, followed by 40 cycles at 95°C for 10 s and 60°C for 30 s. Each sample was run in triplicate for analysis. Melting curve analysis was performed to validate the specificity of each PCR product. The expression levels of the mRNAs and lncRNAs were normalized to the GAPDH level and calculated using the $2^{-\Delta\Delta Ct}$ method.

2.6. *Gene ontology and pathway analysis*

Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were used to determine the potential roles of the differentially expressed mRNAs. GO analysis (*http://geneontology.org/*) provides three-structured networks of defined terms that describe genes and their properties, which includes information on their biological processes, cellular components, and molecular functions. We also adopted the KEGG pathway (*http://www.genome.jp/kegg/*) to predict the biological functions of the target genes.

2.7. *Construction of the lncRNA–mRNA co-expression network*

The lncRNA-mRNA co-expression network was constructed to explore the relationship between the lncRNAs and mRNAs. For each pair of genes, the Pearson correlation coefficient (PCC) was calculated, and the pairs with significant correlations (PCC > 0.90) were chosen to construct the network. Cytoscape Software version 3.4.0 (U.S. National Institute of General Medical Sciences) was used to illustrate the co-expression network.

3. Results

3.1. *Patient characteristics*

Six squamous cervical carcinoma patients who had undergone primary surgical resection between November 2014 and September 2015 were enrolled in this study. The clinical characteristics of the patients are shown in Supplementary Table S1 (*http://www.biosciencetrends.com/action/getSupplementalData.php?ID=18*). All the tumor samples were confirmed to be stage Ib1-IIb squamous cervical carcinomas.

Psychological factors were measured during the pre-surgical clinic visits 1 to 7 days prior to tumor resection. Based on the established threshold of CESD ≥ 16, three participants were determined to show high levels of psychological risk factors. The only measured property that differed significantly between the two groups was the level of depressive scores (CESD, $p = 0.0327$; SDS, $p = 0.0399$).

3.2. *LncRNA and mRNA expression profiles*

Using a 2 or 0.5-fold change as the cut-off, a total of 1,621 lncRNAs were found to be differentially expressed between the high-risk and low-risk groups; of these, 510 were up-regulated and 1,111 were down-regulated in the high-risk group (Supplementary Table S2, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=19*). Table 1 shows the top ten up-regulated and down-regulated lncRNAs. Using the same criteria, we found 1,345 mRNAs showing differential expression between the high-risk and low-risk groups; of these, 325 were up-regulated and 1,020 were down-regulated in the high-risk group (Supplementary Table S3, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=20*). The top ten up-regulated and down-regulated mRNAs are listed in Table 2. The volcano plot of the differentially expressed lncRNAs and mRNAs is shown in Supplementary Figure S1 (*http://www.biosciencetrends.com/action/getSupplementalData.php?ID=21*). Hierarchical clustering analysis was performed to categorize the lncRNAs and mRNAs based on their expression levels in the microarray (Figure 1).

3.3. *Quantitative real-time PCR validation*

To validate the microarray results by real-time PCR

Table 1. Top ten up-regulated and down-regulated lncRNAs in the high-risk group

LncRNAs	Source database	Fold change (up-regulated)	Fold change (down-regulated)	*P*-value
NONHSAT026375	NONCODE v4	83.38818		0.004714203
NONHSAG051449	NONCODE v4	56.622337		0.007290442
NONHSAT006501	NONCODE v4	54.330788		0.028635195
NR_040072.1	RefSeq	29.504402		0.00305648
NR_045603.1	RefSeq	21.908543		0.034243103
NONHSAT029137	NONCODE v4	16.355997		0.001712117
NONHSAT142855	NONCODE v4	15.255895		0.00395211
NONHSAT006296	NONCODE v4	13.86696		0.044070113
NONHSAT026373	NONCODE v4	13.66836		0.049752276
NONHSAT029135	NONCODE v4	12.5354595		0.00199061
NONHSAT097055	NONCODE v4		21.751446	0.008822498
NONHSAT059677	NONCODE v4		20.691536	0.025407365
NONHSAT130141	NONCODE v4		20.616901	0.00182173
TCONS_l2_00010069	broadlincRNA		19.297148	0.001462107
TCONS_l2_00010131	broadlincRNA		18.281479	0.038280915
NONHSAT145020	NONCODE v4		15.2914095	0.004498216
NONHSAT029287	NONCODE v4		15.215009	0.014417922
NONHSAT040550	NONCODE v4		14.950378	0.014418174
NONHSAT026886	NONCODE v4		14.94323	0.002577477
NONHSAG023913	NONCODE v4		14.099488	0.005995478

Table 2. Top ten up-regulated and down-regulated mRNAs in the high-risk group

LncRNAs	Fold change (up-regulated)	Fold change (down-regulated)	*P*-value
SPRR1A	68.54885		0.037800513
A2ML1	47.889496		0.01010738
VTCN1	47.843727		0.012803975
PSCA	38.76277		0.001021038
SH3BGRL2	25.990252		0.01292145
MLPH	20.98717		1.38E-04
POF1B	18.330408		0.03179252
RHCG	17.668486		0.025924051
DNAH14	15.095662		0.011291295
FAM189A2	12.6118145		0.041048728
SLC44A5		63.488415	9.77E-04
CXCL13		22.780071	0.004740034
GLDC		22.185339	0.002045446
CXCL11		18.150942	0.019471126
ELAVL2		14.732332	0.020086708
IFNG		13.957739	0.007875251
KLRC3		12.96415	0.001003543
MMP7		12.581658	0.027674025
UBD		12.484317	0.04345015
IL12RB2		12.357619	0.010752848

quantification, we selected three mRNAs and two lncRNAs from the six cervical cancer samples that were subjected to the microarray analysis. The qRT-PCR results showed that the expression of lncRNA NONHSAT097055 and the CCL5, CXCL9, and HIST1H2AM mRNAs was significantly decreased (p-values for all < 0.05) in the high-risk samples compared to that in the low-risk samples. By contrast, the expression of lncRNA NONHSAT029137 was significantly increased in the high-risk group ($p < 0.05$) (Figure 2). The qRT-PCR results were consistent with those obtained from the microarray analysis, thus confirming the microarray results.

3.4. *Gene enrichment and pathway analysis*

GO analysis was performed for all the differentially expressed mRNAs to identify the potential functions of their coding transcripts. We found that the mRNAs showing significant differential expression between the high-risk and low-risk groups were mainly associated with the immune response (GO:0006955), adaptive immune response (GO:0002250), regulation of immune response (GO:0050776), protein heterodimerization activity (GO:0046982), and transmembrane signaling receptor activity (GO:0004888), which are all involved in various biological processes and molecular functions.

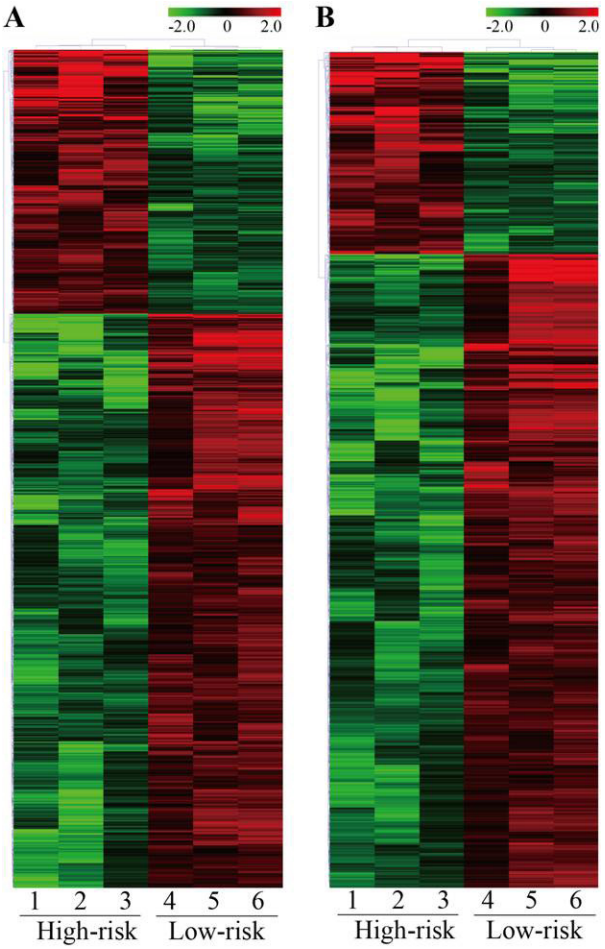

Figure 1. Heat map and hierarchical clustering of the differentially expressed lncRNAs (A) and mRNAs (B) in the high-risk and low-risk groups. The red and green bars indicate the expression levels above and below the relative expression across all the samples.

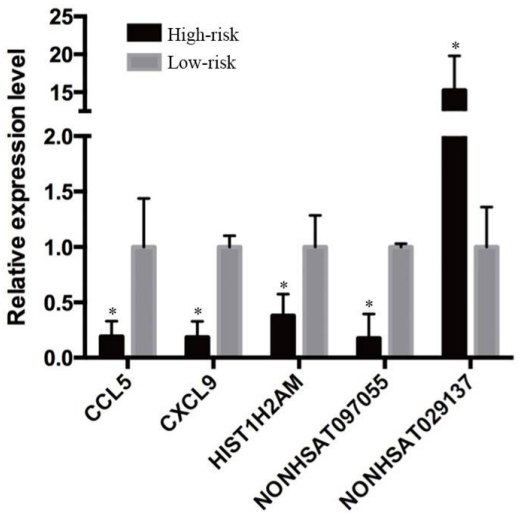

Figure 2. Validation of microarray data using quantitative real-time PCR. Three mRNAs (CCL5, CXCL9, and HIST1H2AM) and two lncRNAs (NONHSAT097055 and NONHSAT029137) were selected and analyzed by qRT-PCR to validate their expression levels. The relative expression level of the target mRNA/lncRNA was normalized, and data displayed in the histograms are expressed as means ± standard deviation (SD), $^{*}p < 0.05$ upon comparison between the high-risk and low-risk groups.

The detailed results are presented in Figures 3 A-C.

KEGG pathway analysis indicated that the most significantly enriched pathways consisted of those that regulate systemic lupus erythematosus (hsa05322), antigen processing and presentation (hsa04612), and natural killer (NK) cell mediated cytotoxicity (hsa04650). The top 20 enriched pathways of the differentially expressed mRNAs are shown in Figure 3 D.

3.5. *Construction of the lncRNA-mRNA co-expression network*

In order to investigate the correlation between the differentially expressed lncRNAs and mRNAs, the lncRNA-mRNA co-expression network was constructed. In total, 509 lncRNAs and 230 mRNAs were included in the co-expression network (Supplementary Table S4, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=22*). To draw the co-expression network, we selected several mRNAs that were found to be involved in the dysregulated functions and pathways in the GO and KEGG pathway analysis. As shown in Figure 4, 52 lncRNAs were found to interact with two mRNAs (CBL and PRKCQ) in the KEGG term of "T cell receptor signaling pathway" (A), 51 lncRNAs were found to interact with two mRNAs (CXCR5 and IL12B) in the KEGG term of "cytokine-cytokine receptor pathway" (B), and 39 lncRNAs were found to interact with two mRNAs (BTLA and ZNF683) in the GO term of "adaptive immune response" (C).

4. Discussion

In this study, we profiled the expression of lncRNAs and mRNAs in cervical cancer samples from patients with different psychological risk levels by microarray analysis. We found that 1,621 lncRNAs and 1,345 mRNAs were differentially expressed between the two groups. GO and KEGG pathway analysis was performed to predict the potential functions of the differentially expressed mRNAs. Moreover, we predicted the target genes of the differentially expressed lncRNAs by constructing lncRNA-mRNA co-expression networks. Based on our results, we predicted that some of the differentially expressed genes play important roles in the psychological stress-induced psychoimmunological effects on cervical cancer.

There are a substantial number of studies on the effects of the chronic activation of the stress response on the immune response associating with cancer initiation and progression (*10*). Psychological stress has been shown to suppress the non-specific and specific components of the immune response, such as antigen presentation, NK cell activity, T cell

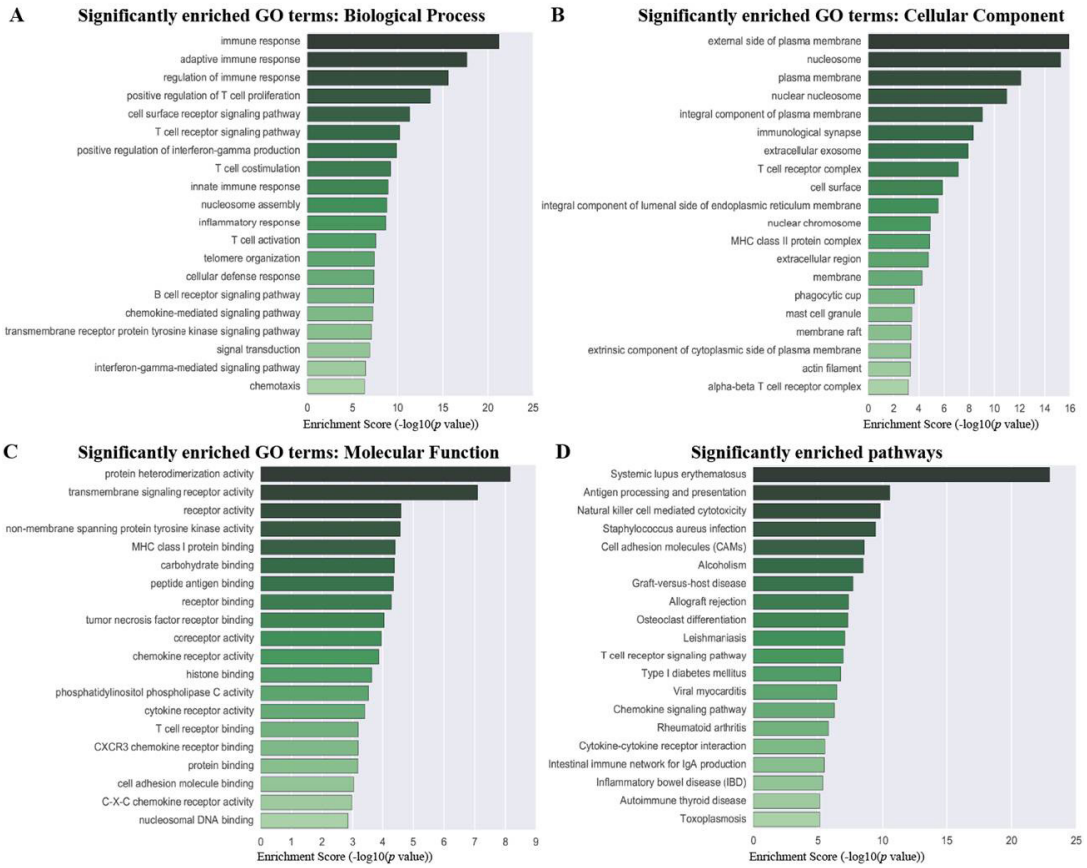

Figure 3. Functional prediction of the differentially expressed mRNAs based on GO enrichment analysis (A-C) and KEGG pathway analysis (D). GO analysis covered three domains: biological processes, cellular components, and molecular functions.

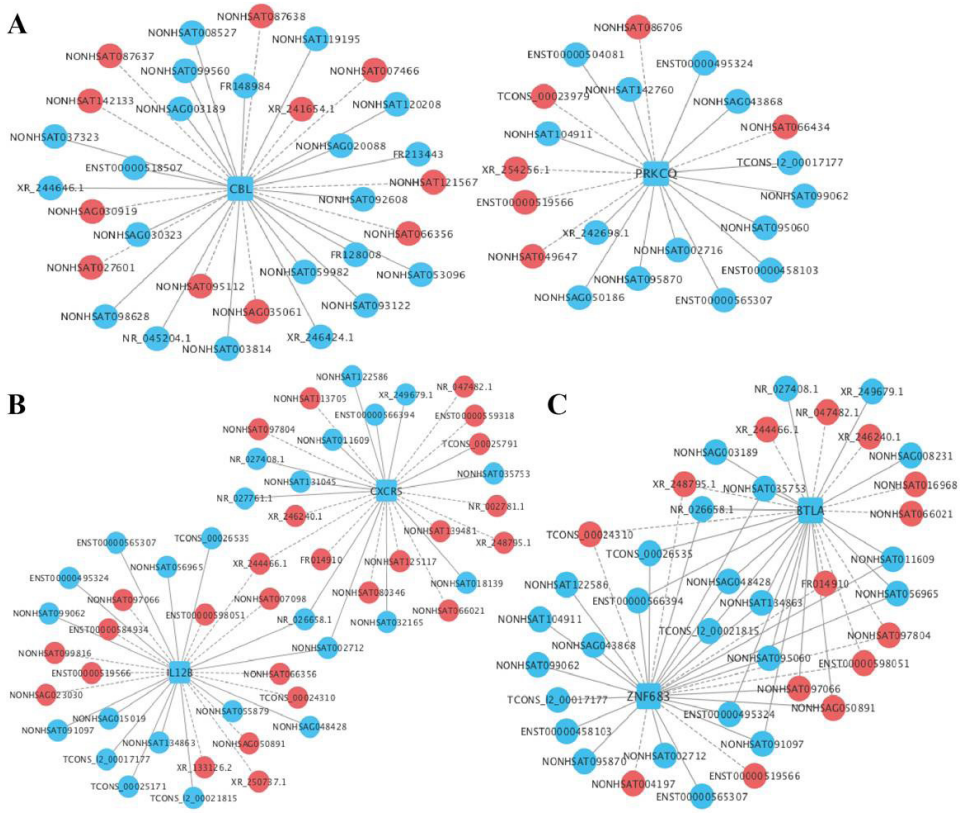

Figure 4. LncRNA–mRNA co-expression network. (A) 52 lncRNAs were found to interact with two mRNAs in the meaningful "T cell receptor signaling pathway". **(B)** 51 lncRNAs were found to interact with two mRNAs in the meaningful "cytokine-cytokine receptor pathway". **(C)** 39 lncRNAs were found to interact with two mRNAs in the GO term of "adaptive immune response". Square nodes represent the mRNAs, and round nodes represent the lncRNAs. The up-regulated and down-regulated genes are indicated in red and blue, respectively. Solid lines indicate a positive correlation, and dashed lines indicate a negative correlation.

proliferation, cytotoxic T cell activity, and production of inflammatory cytokines via mechanisms that involve adrenergic and glucocorticoid-mediated pathways (*7*).

Cancers caused by DNA tumor viruses might be more affected by psychological and immunological factors than those induced by chemical carcinogens (*10*). Researchers have identified persistent infection with a high-risk human papillomavirus (HPV) as the cause of over 99% of cervical cancers (*18*). Fang *et al*. also reported that higher levels of perceived stress are associated with impaired HPV-specific immune response in women with cervical dysplasia (*19*). Moreover, a recent clinical study showed that bereavement is associated with an increased risk of HPV infection and cervical cancer in Sweden (*20*). Stressful life events might also be associated with impaired immune surveillance and possibly poor control over HPV infection and thus increase the risk of cervical cancer.

The ZNF683 (Zinc finger protein 683) and BTLA (B and T Lymphocyte Associated) mRNAs were found to be associated with the GO term "adaptive immune response". ZNF683, also known as Hobit (Homolog of Blimp-1 in T cells), is a transcription factor that mediates a transcriptional program in tissue-resident memory T (Trm) and natural killer T (NKT) cells (*21,22*). Hobit mediates the development and retention of Trm cells and NKT cells in non-lymphoid organs and other nonbarrier tissues and may provide immediate immunological protection against re-infections. BTLA is an immunoglobulin-like molecule belonging to the B7 family, which relays inhibitory signals that suppress the immune response (*23*). Moreover, the interaction between BTLA and its ligand has been reported to be actively involved in the adaptive immune response (*24*). In our study, we constructed a lncRNA-mRNA co-expression network to predict the potential functions of the differentially expressed lncRNAs. Our results showed that several lncRNAs, such as NONHSAT002712, NONHSAT095060, and TCONS_00026535, had significant correlations with ZNF683 and BTLA, suggesting that these lncRNAs had roles in the regulation of the adaptive immune response.

The major limitation of this study is that the data were obtained from a small sample size. In the future, larger studies will be required to establish the generality of these findings. Nevertheless, this study is novel in that it identified genome-wide lncRNAs correlating with psychological stress specifically in cervical cancer tissues and thus provides new insights into the psychological epidemiology of cervical cancer.

Acknowledgements

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