Co-expression Network analysis of LncRNA associated with overexpression of DNMT1 in esophageal epithelial cells

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Research

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Abstract

**Background:** Screening and preliminary identification of DNMT1 high expression related LncRNA, which is involved in various inter-related signaling pathways, has revealed a theoretical basis for various types of disease mechanisms.

**Methods:** LncRNA and mRNA differential expression profiles were identified on a microarray. The 10 LncRNAs with high levels of variation were identified by qRT-PCR. KEGG and GO analyses were used to dissect out differentially expressed mRNAs.

**Results and Conclusions:** Six signal pathways were selected based on the KEGG results of the LncRNA-mRNAs expression network analysis. We found a total of 6987 differentially expressed LncRNAs and 7421 differentially expressed mRNAs were obtained from the microarrays in experimental and control groups ($P<0.05$; Fold Change $>2.0x$). GO analysis KEGG pathway analysis showed high expression of DNMT1 in esophageal epithelial cells. Nine pathways involved mRNA up-regulation including natural killer cell mediated cytotoxicity and many other prominent biochemical pathways. Forty six pathways were associated with down regulated mRNAs and ribosomes involving multiple biological pathways. Co-expression network analysis showed that the 8 mRNA and 16 LncRNA were linked to the P53 signaling pathway. Interactions occurred between 22 LncRNA and 11 mRNA in the ErbB signaling pathway, between 19 LncRNA and 8 mRNA in epithelial cell signal transduction of Helicobacter pylori infection. Interactions were present in 19 LncRNAs and 5 mRNAs in the sphingolipid signaling pathway along with 21 with LncRNA and 12 with mRNAs in the PI3K-Akt signaling pathway. Cytotoxicity interactions occurred with 22 LncRNAs and 9 mRNAs in natural killer cells.

Introduction

Epigenetics is the study of genetic changes in gene activity or function and does not involve changes in the DNA sequence itself. Its molecular mechanisms include DNA methylation, chromosome modification, histone modification and RNA interference. Historically, DNA methylation was discovered in mammals long before the DNA was identified as genetic material [1, 2]. DNA methylation is accomplished by transference of methyl groups from S-adenosylmethionine to the 5' position of cytosine via DNA methyltransferase activity (DNMTs). Three catalytically active DNMTs have been identified in mammals: DNMT1, DNMT3a, and DNMT3b [3]. DNMT1 is the most important enzyme for maintaining DNA methylation status in vertebrates and is also one of the most well-known enzymes. It can play a role in gene silencing as well as DNA methylation repair [4]. High DNMT1 expression levels can cause methylation pattern variations that result in silencing of tumor suppressor genes and oncogene activation. Abnormal DNMT1 activity can lead to prostate[5–7], lung[8, 9], kidney[10–12] and bladder cancer[13–16]. It is clear that the integrity of the DNA methylation system is critical to health in mammals.

Long non-coding RNA (LncRNA) is a form of RNA lacking open reading frames and does not encode proteins. The transcripts are more than 200 bp in length and are found in the nucleus or cytoplasm. A
large number of studies have reported that LncRNA plays an important role in the development of many diseases. It has tissue, cell, developmental, spatio-temporal, and disease related specificity and is widely involved in cell differentiation and metabolism. Cell proliferation occurs in the course of various diseases where normal LncRNA function is altered \(^{17-24}\). Differential expression of LncRNA exhibits tumor specificity, is not affected by other factors, and can be used as an independent tumor specific predictor \(^{25}\). Studies have shown that LncRNA is an important regulatory factor in the human genome that can control DNA methylation and histones as an epigenetic modulator, transcriptional, and post-transcriptional regulator in a cis or trans manner. These activities include modification and chromatin remodeling to silence or activate genes \(^{26-28}\).

Since LncRNA does not encode proteins, it appears to act indirectly as compared to the direct action of mRNA. Therefore co-expression analysis is widely used to elucidate the relationship between LncRNAs and messenger RNA (mRNAs) actions \(^{29,30}\). It can reveal key LncRNAs and help to elucidate new regulatory mechanisms.

In the present study, using a previously developed a high-expression DNMT1 cell line and a normal esophageal epithelial cell line, we used the Agilent Human LncRNA V5 chip technology to screen differentially expressed LncRNA and co-expressing them. Analysis included an in-depth evaluation of specific LncRNA functions that can form a foundation for an in-depth examination of disease mechanisms.

**Methods**

**Samples**

The experimental groups chosen for this study included a highly expression DNMT1 cell line \(^{31}\), which was developed following transfection of a WV0132 plasmid using TALE technology. The control group was a normal esophageal epithelial cell line HEEC.

**LncRNA Microarrays**

The Agilent Human V5 Microarray analysis (Agilent, USA) was performed using a Gene Expression Hybridization Kit (Agilent USA) according to the manufacturer’s instructions. Slides were washed in staining dishes with a Gene Expression Wash Pack (Agilent, USA) and scanned by an Agilent Scanner G2505C (Agilent, USA) with default settings according to the manufacturer’s instructions. Raw data were normalized by the Quantile algorithm using Gene Spring Software 13.1 (Agilent Technologies).

**Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)**

We selected 10 differentially expressed genes to evaluate their activity in overexpressing DNMT1 and esophageal epithelial cells. Total RNA was isolated from all samples using a mirVanaTM RNA Kit
then reverse transcribed using a Quick Amp Labeling Kit, One-Color (Agilent, USA) according to the manufacturer’s instructions. qRT-PCR was performed using a QuantiFast® SYBR® Green PCR Kit (Qiagen, Germany). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Sequencing primers are shown below (Table 2).

| Prime name                  | Forward          | Reverse             |
|-----------------------------|------------------|---------------------|
| Inc-OR1M1-1: 1              | CTGACATTTCAAGAGGTTGGA | TGACTGATTCACTATTTGGGTGC |
| Inc-IGFBP3-1: 1             | CTTCCTGGAGAGTCACCTCCTA | AGCCTTTCAAGAGGATACTCG |
| NR_003367(PVT1)             | TTTCAGCACTCTGGAGGGG | AACACAGAGCACAAGGAC |
| ENST00000505089             | CATCCTGATACCAAAGGCT | TTGATGTTGCTGGATTTGCG |
| ENST00000568998             | CAAGGCTCCTCATAAGCA | GCACCTTTGAGGATGCAAT |
| Inc-ST8SIA4-8: 1            | ATGGTGACGTGATGTAATGC | TCTGAGCGGATAAATGGGACT |
| Inc-ZNF530-1: 1             | CGACCCAGGTATTATTGAGTG | TCAAACCTTGGGCTCAAGG |
| NR_110492(TUG1)             | TGGCTATTTGATGGGGTG | TGACTGATGTCCTGACCG |
| NR_002819(MALAT1)           | CCTAAGGTCAGGAGAGTGC | GGTACCTGCAAGCATTCCCTC |
| ENST00000436710             | CTTTGTCTTGTGGTACACC | AGAACTTTCTCCACAGG |
| GAPDH                       | TGTTGCCATCAATGACCCCTT | CTCCAGCAGTGACTCACCG |

### Statistical Analysis

Data were analyzed using SPSS (version 17.0; SPSS Inc., Chicago, IL, USA). Differentially expressed genes or LncRNAs were then identified by fold change as well as P value levels calculated by t-test. The threshold set for up- or down-regulated genes was \( \geq 2.0 \) times the median value and a P value of \( \leq 0.05 \). LncRNAs-mRNA co-expression networks were constructed using Cytoscape software54 (version 3.4.0; The Cytoscape Consortium, San Diego, CA, USA).

### Results

#### Identification of differentially expressed LncRNAs and mRNAs

There were 6987 LncRNAs that were differentially expressed from the microarrays of experimental and control groups. Of those, 3654 that were up-regulated and 3333 were down-regulated. In addition, 7421 differentially expressed mRNAs were identified that included 2254 that were up-regulated and 5167 that
were down-regulated. A volcano plot was created to identify differences among the various LncRNAs (Fig. 1).

We also randomly selected 10 differentially expressed genes and further performed quantitative real-time polymerase chain reaction (qRT-PCR) to examine their expression levels (Table 1). The resulting melting curves all showed single peaks, with PCR amplification to show greater specificity (Fig. 2–1 to 2–11).

| LncRNA                     | Experimental group | Control group | T   | P       |
|----------------------------|--------------------|---------------|-----|---------|
| ENST00000436710            | 0.19 ± 0.18        | 1.48 ± 0.74   | 2.93| 0.043   |
| PVT1                       | 1.73 ± 0.25        | 1.01 ± 0.21   | 3.80| 0.019   |
| TUG1                       | 0.12 ± 0.12        | 1.05 ± 0.38   | 4.07| 0.015   |
| MALAT1                     | 0.09 ± 0.003       | 1.01 ± 0.20   | 8.20| 0.015   |
| ENST00000505089            | 0.04 ± 0.04        | 1.08 ± 0.50   | 3.61| 0.023   |
| Inc-OR1M1-1: 1             | 19.21 ± 8.97       | 1.02 ± 0.27   | 3.51| 0.072   |
| Inc-ST8SIA4-8: 1           | 0.12 ± 0.04        | 1.02 ± 0.28   | 5.53| 0.005   |
| Inc-ZNF530-1: 1           | 3.37 ± 0.43        | 1.05 ± 0.43   | 6.60| 0.003   |
| Inc-IGFBP3-1: 1           | 3.47 ± 0.35        | 1.00 ± 0.09   | 11.90| 0.000   |
| ENST00000568998            | 0.19 ± 0.05        | 1.00 ± 0.09   | 13.47| 0.000   |

**GO analysis and KEGG analysis**

Gene ontology (GO) analyses was conducted to explore the function of the 7421 differentially expressed mRNAs. The results showed that there are 1825 up-regulated mRNAs expressed during various biological processes including those involved in blood coagulation, type I interferon signaling pathways and response to viruses (Fig. 3A). There were 3483 down-regulated mRNAs in biological processes such as those involving viruses, SRP-dependent co-translational proteins targeting membrane and gene expression (Fig. 3B). In terms of cellular components, there were 1937 up-regulated mRNAs expressed including those associated with the extracellular space, the cell surface and the extracellular membrane (Fig. 3C). There were 3655 down-regulated mRNAs expressed including those involved with cellular components such as the cytoplasm, nucleoplasm and cytosol (Fig. 3D). Evaluating molecular function, there were 1817 up-regulated mRNAs detected that included those involved in protein homodimerization activity, heparin binding and SH3 domain binding (Fig. 3E). There were 3517 down-regulated mRNAs expressed involving cellular components that included protein binding, poly (A) RNA binding and ligase activity (Fig. 3F).
KEGG pathway analysis was conducted to examine the function of the 7421 differentially expressed mRNAs. The results show that up-regulated mRNAs were highly enriched in 9 gene pathways, including natural killer cell mediated cytotoxicity, and glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate and steroid biosynthesis (Fig. 4A). Down-regulated mRNAs were expressed in the 46 gene pathways including those involving ribosomes, pancreatic cancer and the ErbB signaling pathway (Fig. 4B).

**LncRNA-mRNA co-expression networks**

Based on the KEGG pathway results, we selected 6 pathways from the down-regulated mRNA signaling and the up-regulated mRNA signaling pathways to perform co-expression network analysis. In particular, we examined the P53 signaling and ErbB signaling pathway respectively as well as epithelial cell signaling in Helicobacter pylori infection, sphingolipid signaling pathway, PI3K-Akt signaling pathway, and natural killer cell mediated cytotoxicity. Our results showed that 16 LncRNAs interacted with 8 mRNAs in the P53 signaling pathway (Fig. 5A), 22 LncRNAs interacted with 11 mRNAs in the ErbB signaling pathway (Fig. 5B), 19 LncRNAs interacted with 6 mRNAs in epithelial cell signaling in Helicobacter pylori infection (Fig. 5C), 19 LncRNAs interacted with 5 mRNAs in the sphingolipid signaling pathway (Fig. 5D), 21 LncRNAs interacted with 12 mRNAs in PI3K-Akt signaling pathway (Fig. 5E), and 22 LncRNAs interacted with 9 mRNAs in Natural killer cell mediated cytotoxicity (Fig. 5F).

**Discussion**

DNMT1 can regulate the expression of genes in many different, complex ways. It mediates DNA methylation, modification of histones, and chromosome remodeling. It constitutes a very complex epigenetic regulatory network and regulates gene co-expression. In normal tissues, CpG islands in the gene promoter region are generally unmethylated. In tumor cells, the opposite is often true where CpG islands are hypermethylated which leads to silencing of their related genes[^32,33]. During replication, DNMT1 is localized in the replication complex and is associated with a methylated CpG island site in the parental chain which catalyzes the methylation gene then adds it to the corresponding CpG island site on the daughter strand. Validating DNMT1 location in differentiated cells allows comparison with the original methylation profile[^34]. Studies have shown that hypermethylated genes are found in breast, colon, and stomach cancers[^35–37]. Generally, DNMT1 expression increases before DNA methylation, which may cause abnormal DNA methylation.

LncRNAs have been the focus of a number of studies in recent years and have been found to be associated with the development of many types of tumors involving epigenetic, transcriptional, and post-transcriptional regulation during gene expression. While regulating DNA methylation, LncRNA mainly affects the expression of related genes by altering the methylation levels of CpG islands in the genes promoter region. Therefore, understanding LncRNA differential expression in the DNMT1 high-expression cell line and in normal esophageal epithelial cells line may be useful for understanding its function.
During co-expression analysis of the P53 signaling pathway, we found that LncRNA TUG1 is associated with the mRNA CDKN2A. TUG1 is widely expressed in various tumors and exhibits a high expression levels in nervous system tumors, colorectal cancer, hematological system tumors, and bladder cancer. However, the expression level of TUG1 varies with different tissue types. It was found that compared with normal lung tissues/cells and para-cancerous tissues, the expression of TUG1 in non-small cell lung cancer tissues or cells was significantly reduced. This suggests that, on the one hand, TUG1 may play a cancer-promoting role but and it can also play a role in inhibiting cancer\[^{38}\]. In the present study we found that TUG1 showed low expression levels in DNMT1 high-expression cells suggesting that TUG1 may be a tumor suppressor in this system. Khalil et al. \[^{39}\] has demonstrated, using co-immunoprecipitation, that TUG1 recruits and binds to polycomb repressive complex 2 (PRC2) and PRC2 catalyzing the dimethylation of histone H3 at position 27. Trimethylation of lysine occurs at residue27 of histone3, H3 K27 me3 which, in turn, affects miRNAs, cyclin-dependent kinase inhibitors (eg p15, p16, p21, p27, p57) and blood vessels which activate expression of related genes that participate in tumor development. CDKN2A is a cyclin dependent kinase inhibitor that is located on human chromosome 9p21 and encodes two different proteins. One is a cell cycle dependent kinase inhibitor p16\(^{INK4α}\) which is encoded by exons 1α, 2 and 3. The other is alternate reading frame (ARF), encoded by exons 1β, 2 and 3 (in mice, called p19\(^{ARF}\)), both of which are cellular regulators through cyclinD-CDK4-pRb-E2F and MDM2 respectively.

The p53 pathway is involved in cell cycle regulation \[^{40}\]. Therefore, it may be inferred that TUG1 inhibits CKIs by recruiting PRC2 leading to excessive cyclinD-CDK4/6 kinase activation which may disrupt the cell cycle and promote cell proliferation. The loss of p16\(^{INK4α}\) leads to excessive activation of CDK4/6 kinase, however, modulation of the p16\(^{INK4α}\)/pRb pathway will not inhibit the cancer. Much of this discussion, however, is speculative at this point and will require further verification both \textit{in vivo} and \textit{in vitro}.

LncRNA PVT1 was found to be related to CDKN2A mRNA in the ErbB signaling pathway. Further analysis of the function of LncRNA was provided by GO analysis. In addition, 19 LncRNAs interacted with 6 mRNAs in epithelial cell Helicobacter pylori infection signaling, 19 LncRNAs interacted with 5 mRNAs in the sphingolipid signaling pathway, 21 LncRNAs interacted with 12 mRNAs in PI3K-Akt signaling pathway and 22 LncRNAs interacted with 9 mRNAs in Natural Killer cell mediated cytotoxicity. Therefore, our results suggest a key pathogenic role for LncRNAs.

**Conclusion**

This study does have some limitations. The results were obtained from the bioinformatic analysis and microarray analysis. Therefore, further mechanistic studies will be needed to confirm the role of these differentially expressed genes and pathways. This database study does, however, provide a valuable catalyst for further study.

**Abbreviations**

DNMTs
DNA methyl transferase
LncRNA
Long non-coding RNA
KEGG
Kyoto Encyclopedia of Genes and Genomes
GO
Gene Ontology
PRC2
polycomb repressive complex 2
qRT-PCR
Real-time Polymerase Chain Reaction

Declarations

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Conflict of Interest

The author(s) declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Other statements

The study didn't involve studies involving human participants and animals. And all authors were aware of and agreed to submit the article.

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