Research Article

Inference of Crosstalk Effects between DNA Methylation and lncRNA Regulation in NSCLC

Binhua Tang 1,2

1 Epigenetics & Function Group, School of the Internet of Things, Hohai University, Jiangsu 213022, China
2 School of Public Health, Shanghai Jiao Tong University, Shanghai 200025, China

Correspondence should be addressed to Binhua Tang; bh.tang@outlook.com

Received 12 February 2018; Revised 23 April 2018; Accepted 8 May 2018; Published 24 June 2018

1. Introduction

Comprehensive interrogation and genome-wide annotation of intercellular differential DNA methylation and its association in long noncoding RNAs (lncRNA) regulation are open questions in carcinoma epigenetics studies [1–3].

Genome-wide DNA methylation analysis across multiple samples, especially the recent pan-cancer study can retrieve cell- and tissue-specific characteristics with identification of differentially methylated loci and regions [4–7].

Increasing evidences highlight lncRNA as crucial regulators with essential cancer cell functions in cell proliferation, apoptosis, and metastasis. LncRNAs can regulate transcription and posttranscription through diverse biological mechanisms in carcinogenesis [8–11].

Till now, several tools and databases were proposed concerning functional curation and analysis of lncRNA roles in cancer and disease, for example, NONCODE [12], Lnc2Cancer [13], and LncRNADisease [14], but there still lacks systematic investigation of lncRNA regulatory activities under other epigenetic contexts, such as DNA methylation, in development and progression of lung carcinoma.

Currently, NSCLC (non-small-cell lung carcinoma) accounts for more than 85% lung carcinoma occurrences, and NSCLC is relatively insensitive to clinical chemotherapy, compared to small cell carcinoma [15–17]. Herein based on 24 group-wise (paired) NSCLC patient tissue samples (12 carcinoma versus 12 paracarcinoma), we integrate the corresponding lncRNA and DNA methylation profiling information to study the underlying regulatory mechanisms by our developed toolkit, MetLnc.

MetLnc is designed for integrating lncRNA and Human Methylation 450K Beadchip assay information [18–20], together with the suitable format from such platform as the Reduced Representation Bisulfite Sequencing (RRBS) profiling [7, 21–23]. MetLnc can implement intercellular interrogation of lncRNA and DNA methylation status among multiple samples, perform statistical analysis on methylated CpG loci and regions, and yield integrative visualization for the analysis results.

Together our developed toolkit performs benchmark analyses with the group-wise NSCLC patient tissue profiling and TCGA cohort resources, thus it is proved to be a versatile analytic approach in carcinoma epigenetics study.
2. Materials and Methods

2.1. Structure and Function Composed in MetLnc. The function and analysis flow in MetLnc are depicted in Figure 1, which covers main steps in processing DNA methylation and lncRNA profiling information, differentially expressed loci annotation and multi-source omics integration.

MetLnc is designed to carry out three main functional processes, namely, (I) DNA methylation and lncRNA profiling preprocess; (II) epigenetic information retrieval, including CpG annotation, differentially methylated CpG loci, and differential lncRNA candidates; and (III) knowledge integration and discovery, genome-wide comparison, and association analysis on the methylation and lncRNA effects on regulation. And we deposited the toolkit on GitHub (github.com/gladex/MetLnc/) under GNU General Public License v3.0.

2.2. NSCLC Sample Source for Profiling Experiments. The profiling experiments were carried out on the group-wise 12 tumor and 12 paratumor NSCLC tissues, by the collaborated lab in a NSCLC pilot project.

After the preprocessing and purification on the sample cells, DNA methylation and lncRNA experiments further profiled the corresponding methylation level and lncRNA expression level to investigate the crosstalk effects between the two epigenetic factors.

DNA methylation status profiling was implemented using Illumina Infinium HumanMethylation450 BeadChip platform for 24 paired NSCLC patient samples (carcinoma versus paracarcinoma tissues). lncRNA profiling was carried out based on Illumina HiSeq-2000 RNA Sequencing platform.

2.3. Analysis of RNA Sequencing-Based lncRNA Profiling. The corresponding RNA-seq profiling analysis was implemented to identify totally 124,060 differentially expressed non-coding RNA (ncRNA) candidates, with the log2 fold change range [-8.551009, 7.349755] and the ncRNA length range from 10 bp to 1,699,000 bp, respectively. We further determined 1,468 significant differentially expressed lncRNAs with the length ≥ 200 bp, the log2 fold change ≥ 2, and corresponding adjusted p-value ≤ 0.05. Then the lncRNA targets were annotated with UCSC gene information for further downstream analysis.

2.4. DNA Methylation Distribution for Paired NSCLC Patient Tissues. We detected DNA methylation level for the group-wise NSCLC patient tissues utilizing Illumina Infinium HumanMethylation 450 BeadChip technique. Totally 485,577 probes were identified from the pairwise carcinoma (12 patient tissues) and paracarcinoma (12 patient tissues) group. After necessary preprocess, total 6,163 probes were determined as significant hyper-/hypo- differentially methylated CpG sites (SDMC), with the absolute methylation difference percentage ≥ 0.25 and corresponding adjusted p value ≤ 0.05. And we further annotate those 6,163 significantly differentially methylated CpGs with UCSC genome information (including SNP promoter, and enhancer annotation), together with CpG island (shore) information. Thus, we found there are 723 out of 6163 (11.73%) SDMCs overlap with the known SNP annotation, 4,947 (80.27%) SDMCs are identified from CpG island/shores regions, 1,693 (27.47%) SDMCs locate at enhancer regions, and 1,230 (19.96%) SDMCs are detected from DHS.

2.5. Integration of DNA Methylation and lncRNA Profiling Information. After the preprocessing DNA methylation and lncRNA profiling data, we further integrated both information based on the genomic location, namely, considering differentially methylated loci within promoter region (TSS±1000 bp) and body region of differentially expressed lncRNA candidates.

Together we further identified the potential targeted genes based on the cis-regulation of lncRNAs; namely, we only consider targeted genes where their promoter region (TSS±1000 bp) has differentially expressed lncRNAs.

3. Comprehensive Analysis and Functional Annotation

3.1. Differential Analysis for Paired NSCLC Patient Tissues. We further studied the crosstalk mechanism of DNA methylation and lncRNA on transcription regulation. We performed the profiling experiments on the group-wise 12 tumor and 12 paratumor NSCLC tissues.

After the sample normalization and necessary data preprocessing, we investigated their differential expression status of lncRNA and DNA methylation and performed meta-analysis on the identified candidates with coexpression activities comprehensively; see Figure 2.
Differential expression and annotation analysis on both DNA methylation and IncRNA identified that 6,163 significantly differentially methylated CpGs have 1,339 targeted genes, and 1,468 significant differentially expressed IncRNAs have 2,035 targeted genes, Figures 2(a) and 2(b).

Within the two targeted gene groups, there are 164 common targeted genes. Inside the Circos diagram, Figure 2(c), each arc represents a gene list, where each gene has a spot on the arc. Dark orange color represents the genes that appear in multiple lists and light orange color represents genes that
are unique to that gene list. Purple lines link the same genes shared by multiple gene lists.

Through the Gene Ontology analysis on the differentially expressed candidates, we found that DNA methylation has the overwhelming effects on the entire regulation process, compared with IncRNA, although DNA methylation has the relatively lower targeted genes (1,339) than IncRNA (2,035), Figure 2(d); and the ontology enrichment network with its nodes displayed as pies, and the pie sector is proportional to the number of hits originated from a gene list related to DNA methylation (red) or IncRNA (blue), respectively.

We further interrogated the ontology distribution for the 164 common candidate genes regulated by DNA methylation and IncRNA and found their ontology terms cover the below processes that can be clustered into four major groups, namely, (I) negative regulation of development (multicellular organismal process/cell differentiation); (II) epithelial cell development (differentiation); (III) (trans-)synaptic signaling/synaptic transmission; (IV) muscle organ/tissue development.

The above phenomena indicate that DNA methylation and IncRNA in NSCLC mainly initiate the crosstalk activities in development and differentiation stages at epithelial cell and synaptic levels. We wonder whether such clues have any linkage with clinical outcomes in lung carcinoma.

3.2. Clinical Outcome by the Crosstalk between DNA Methylation and IncRNA. Thus, we retrieved the corresponding LUAD (Lung Adenocarcinoma, with 506 patient cohort) and LUSC (Lung Squamous Cell Carcinoma, with 495 patient cohort) clinical information from TCGA (The Cancer Genome Atlas) resources [5, 17] and further interrogated the clinical outcome association for the differentially expressed candidate genes, which are putatively regulated by DNA methylation and IncRNA.

Furthermore, to avoid the heterogeneity problem within tissue samples, we utilized z-score method to normalize the TCGA expression data and selected z-score $= \pm 1.96$ as the differential expression threshold; thus, on each gene candidate, we can further associate its differential expression status with clinical outcome by means of Kaplan-Meier survival analysis approach [24, 25].

For simplicity, we selected four representative candidate genes, namely, NTM (11q25, Entrez:50863), SIGLEC12 (19q13.41, Entrez:89858), TNXB (6p21.32, Entrez:7148), and LIMCH1 (4p13, Entrez:22998) as study cases.

Figure 3 illustrates the analysis results for LUAD (top) and LUSC (bottom) cases with calculated log-rank test p-values at the bottom left panel, respectively.

Comparatively, we found that NTM can act as clinical signature for both LUAD and LUSC, the entry of 488 patients...
with not altered status has better clinical outcomes than the altered entry; SIGLEC12 also works as signature for LUAD and LUSC, but the entry in LUAD with altered status has worse clinical outcome than that in LUSC; similarly, for TNXB, the entry in LUAD with altered status has worse clinical outcome; and LIMCH1 in LUSC indicates a better outcome for 487 patients with altered status.

Generally, the analyses reveal that the four gene candidates can work as potential lung cancer biomarkers, and TNXB and LIMCH1 are specifically to LUAD and LUSC, respectively.

4. Discussion and Conclusion

Intercellular crosstalk effects between DNA methylation and lncRNA regulation remain open questions in carcinoma epigenetics, although both epigenetic regulations are commonly regarded as important factors in cell differentiation and development processes.

We present an application toolkit MetLnc in integration and annotation for DNA methylation and lncRNA, thus to comprehensively analyze differentially methylated loci (regions) and lncRNA transcription through deep interrogation. MetLnc provides multiple versatile functions for investigating and annotating DNA methylation profiles. Generally, the toolkit can act as a comprehensive approach on epigenetic omics integration and deep interrogation.

Furthermore, through benchmark with NSCLC tissue profiling resources, we interrogate differentially methylated CpG loci and lncRNA as clues for inferring crosstalk effect between DNA methylation and lncRNA regulation.

Together via the clinical association analysis with TCGA cohort resources, we further determined the clinical outcome association for the identified differentially expressed genes, which are putatively regulated by DNA methylation and lncRNA. We found the identified gene candidates can work as potential lung cancer biomarkers; especially TNXB and LIMCH1 have clinical survival specificity to LUAD and LUSC, respectively. Such discovery has meaningful implications to cancer epigenetics and further clinical trial studies.

Abbreviations
LncRNA: Long non-coding RNA
NSCLC: Non-small-cell lung carcinoma
LUAD: Lung adenocarcinoma
LUSC: Lung squamous cell carcinoma
TCGA: The Cancer Genome Atlas.

Data Availability
The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure
The abstract was previously posted in ISB 2017.

Conflicts of Interest
The author declares that there are no conflicts of interest.

Acknowledgments
This work was supported by the Natural Science Foundation of Jiangsu, China (BE2016655 and BK20161196), and the Fundamental Research Funds for China Central Universities (2016B08914). This work made use of the resources by the Open Cloud Consortium- (OCC-) sponsored project, supported in part by grants from Gordon and Betty Moore Foundation and the National Science Foundation (USA) and major contributions from OCC members.

References
[1] V. N. Kristensen, O. C. Lingjærde, H. G. Russnes, H. K. M. Vollan, A. Frigessi, and A.-L. Barresen-Dale, "Principles and methods of integrative genomic analyses in cancer," *Nature Reviews Cancer*, vol. 14, no. 5, pp. 299–313, 2014.
[2] The Cancer Genome Atlas Network, "Comprehensive molecular characterization of human colon and rectal cancer," *Nature*, vol. 487, no. 7407, pp. 330–337, 2012.
[3] A. Meissner, T. S. Mikkelson, H. Gu et al., "Genome-scale DNA methylation maps of pluripotent and differentiated cells," *Nature*, vol. 454, no. 7205, pp. 766–770, 2008.
[4] T. Witte, C. Plass, and C. Gerhauser, "Pan-cancer patterns of DNA methylation," *Genome Medicine*, vol. 6, no. 8, article 66, pp. 1–18, 2014.
[5] The Cancer Genome Atlas Research Network, J. N. Weinstein, E. A. Collisson et al., "The cancer genome atlas pan-cancer analysis project," *Nature Genetics*, vol. 45, no. 10, pp. 1113–1120, 2013.
[6] M. D. M. Leiserson, F. Vandin, H.-T. Wu et al., "Pan-cancer network analysis identifies combinations of rare somatic mutations across pathways and protein complexes," *Nature Genetics*, vol. 47, no. 2, pp. 106–114, 2015.
[7] B. Tang, Y. Zhou, C. Wang, T. H. Huang, and V. X. Jin, "Integration of DNA methylation and gene transcription across nineteen cell types reveals cell type-specific and genomic region-dependent regulatory patterns," *Scientific Reports*, vol. 7, no. 1, 2017.
[8] Z. Du, T. Fei, R. G. W. Verhaak et al., "Integrative genomic analyses reveal clinically relevant long noncoding RNAs in human cancer," *Nature Structural & Molecular Biology*, vol. 20, no. 7, pp. 908–913, 2013.
[9] F. Li, Y. Xiao, F. Huang et al., "Spatiotemporal-specific lncRNAs in the brain, colon, liver and lung of macaque during development," *Molecular Biosystems*, vol. 11, no. 12, pp. 3253–3263, 2015.
[10] A. M. Schmitt, J. T. Garcia, T. Hung et al., "An inducible long noncoding RNA amplifies DNA damage signaling," *Nature Genetics*, vol. 48, no. 11, pp. 1370–1376, 2016.
[11] Z. T. Beck, Z. Xing, and E. J. Tran, "LncRNAs: Bridging environmental sensing and gene expression," *RNA Biology*, vol. 13, no. 12, pp. 1189–1196, 2016.
[12] Y. Zhao, H. Li, S. Fang et al., "NONCODE 2016: an informative and valuable data source of long non-coding RNAs," *Nucleic Acids Research*, vol. 44, no. 1, pp. D203–D208, 2016.
[13] S. Ning, J. Zhang, P. Wang et al., “Lnc2Cancer: a manually curated database of experimentally supported lncRNAs associated with various human cancers,” *Nucleic Acids Research*, vol. 44, no. 1, pp. D980–D985, 2016.

[14] G. Chen, Z. Wang, D. Wang et al., “LncRNADisease: a database for long-non-coding RNA-associated diseases,” *Nucleic Acids Research*, vol. 41, no. 1, pp. D983–D986, 2013.

[15] J. Botling, K. Edlund, M. Lohr et al., “Biomarker discovery in non-small cell lung cancer: integrating gene expression profiling, meta-analysis, and tissue microarray validation,” *Clinical Cancer Research*, vol. 19, no. 1, pp. 194–204, 2013.

[16] J. D. Campbell, A. Alexandrov, J. Kim et al., "Distinct patterns of somatic genome alterations in lung adenocarcinomas and squamous cell carcinomas," *Nature Genetics*, vol. 48, no. 6, pp. 607–616, 2016.

[17] Cancer Genome Atlas Research Network, “Comprehensive genomic characterization of squamous cell lung cancers,” *Nature*, vol. 489, no. 7417, pp. 519–525, 2012.

[18] J. Maksimovic, L. Gordon, and A. Oshlack, “SWAN: subset-quantile within array normalization for illumina infinium human-methylation450 beadchips,” *Genome Biology*, vol. 13, no. 6, 2012.

[19] M. Bibikova, B. Barnes, C. Tsan et al., "High density DNA methylation array with single CpG site resolution," *Genomics*, vol. 98, no. 4, pp. 288–295, 2011.

[20] R. Lister, M. Pelizzola, R. H. Dowen et al., "Human DNA methylomes at base resolution show widespread epigenomic differences," *Nature*, vol. 462, no. 7271, pp. 315–322, 2009.

[21] A. Meissner, A. Gnirke, G. W. Bell, B. Ramsahoye, E. S. Lander, and R. Jaenisch, "Reduced representation bisulfite sequencing for comparative high-resolution DNA methylation analysis," *Nucleic Acids Research*, vol. 33, no. 18, pp. 5868–5877, 2005.

[22] H. Guo, P. Zhu, L. Yan, R. Li, B. Hu, and Y. Lian, "The DNA methylation landscape of human early human embryos," *Nature*, vol. 511, no. 7511, pp. 606–610, 2014.

[23] A. Kundaje, W. Meuleman, and J. Ernst, "Integrative analysis of 111 reference human epigenomes," *Nature*, vol. 518, pp. 317–330, 2015.

[24] M. J. van de Vijver, Y. D. He, L. J. van 'T Veer et al., "A gene-expression signature as a predictor of survival in breast cancer," *The New England Journal of Medicine*, vol. 347, no. 25, pp. 1999–2009, 2002.

[25] B. Tang, H.-K. Hsu, P.-Y. Hsu et al., "Hierarchical modularity in EKre transcriptional network is associated with distinct functions and implicates clinical outcomes," *Scientific Reports*, vol. 2, article 875, 2012.