Original Paper

Analysis of Long Non-Coding RNA Expression of Lymphatic Endothelial Cells in Response to Type 2 Diabetes

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Key Words

Lymphatic endothelial cells • Type 2 diabetes • Long non-coding RNA • H19

Abstract

Background: Recent evidence has indicated that long non-coding RNA (lncRNA) is involved in the pathogenesis of type 2 diabetes, but nothing is known about lncRNA expression changes of lymphatic endothelial cells in response to type 2 diabetes. **Methods:** The GSE38396 dataset was downloaded from the Gene Expression Omnibus database and the probe sets of Human Genome U133 Plus2.0 microarray were annotated for lncRNA. Differentially expressed lncRNAs between diabetic and non-diabetic lymphatic endothelial cells were calculated. **Results:** Compared with lymphatic endothelial cells in non-diabetic patients, 31 lncRNAs were down-regulated and 79 lncRNAs were up-regulated in lymphatic endothelial cells of type 2 diabetic patients. Several known lncRNAs were found, such as H19, GAS5, UCA1, CRNDE, GAS5, and LINC00312. Co-expression network of differentially expressed lncRNAs and mRNAs were constructed. Based on genomic regions of these lncRNAs, we found that binding sites of MAF and TCF3 were enriched and these lncRNAs may be related to insulin receptor signaling pathway and response to insulin stimulus. **Conclusions:** In a word, we found a set of lncRNAs were differentially expressed in lymphatic endothelial cells in response to type 2 diabetes and these lncRNAs may be involved in the pathogenesis of diabetes-related complications.

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Introduction

The incidence of type 2 diabetes has been rapidly increasing worldwide [1]. Type 2 diabetes is a complex endocrine and metabolic disorder and leads to significant adverse effects to human health. Diabetes impairs the quality of life of many people mainly thorough the cardiovascular complications [2, 3]. Dysfunction of endothelial cells is crucial for the pathogenesis of diabetic vascular diseases [4, 5].

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In mammals, there are two specialized vascular systems: the blood vasculature and the lymphatic vasculature and these two networks are highly related [6]. Type 2 diabetes leads to microvascular lesions and causes ischemia [7]. The blood vascular endothelial cells show imbalances of secretion of pro- and anti-inflammatory cytokines and increased prothrombotic activity [8]. However, little is known about the involvement of lymphatic endothelial cells in human type 2 diabetes.

Long non-coding RNAs (lncRNA) was considered as “transcription noise”, but mounting papers have demonstrated that lncRNA transcripts are widely involved in almost every aspects of cell biological function, such as cell differentiation, metabolism, and apoptosis [9, 10]. Also, recent evidence has proved that lncRNA are also important for the biological function of endothelial cells and lncRNA expression profile is altered in response to stimulus or hyperglycemia in endothelial cells [10-14]. But nothing is known about the lncRNA profile alteration of lymphatic endothelial cells in type 2 diabetes [15, 16].

In this article we reported dysregulation of many lncRNAs, including H19, CRNDE, and TUG1 in lymphatic endothelial cells in human type 2 diabetes. It is predicted by bioinformatics methods that these altered lncRNA may be involved in the response to insulin stimulus.

Materials and Methods

Processing of Lymphatic endothelial cells

The detailed methods were described by Haemmerle M [17]. Briefly, skin samples (n = 4 in each group) were taken from the proximal region of amputated legs or abdominoplastic tissue, and care was taken to excise areas at maximal distance from inflammatory or ulcerous changes (15 cm). Then, human skin was dermatomized and epidermis and dermis dislocated by incubation in dispase solution. Lymphatic endothelial cells were labeled and isolated with antibodies in a three-step procedure. Total RNA of lymphatic endothelial cells were extracted and amplified and then hybridized to Affymetrix Human Genome U133 (HGU133) Plus 2.0 arrays.

Microarray data processing

The raw microarray data was downloaded from the gene expression omnibus (GEO) database and this dataset could be accessed by the accession number GSE38396. In accordance with Haemmerle M [17], the raw files of first normalized using the log scale robust multi-array analysis with default settings. After normalization, expression value of each probe was obtained. And differentially expressed probe sets between diabetic vs. non-diabetic lymphatic endothelial cells were calculated using significant analysis of microarray software. $P < 0.05$ was considered as statistically significant. Heatmap of differentially expressed lncRNAs was generated by Cluster (V3.0) software.

lncRNA annotation

The HGU133 Plus2.0 microarray was used in this study and this array is widely used by numerous researchers in almost every kind of human tissues or cells. the HG U133 plus2.0 array has been annotated for lncRNA to probe lncRNA expression in cancers. In this study, we used the annotation by Zhang X [18]. Only probes that were annotated as lncRNA were selected and transcript ID, chromosome location, strand, biologic types, and other annotation information were also downloaded.

lncRNA-mRNA co-expression network

We built lncRNA-mRNA-network to identify the interactions between mRNA and lncRNA. lncRNA-mRNA network was built according to the normalized signal intensity of specific expression of mRNA and lncRNA. For each pair of mRNA-lncRNA, mRNA-mRNA or lncRNA-lncRNA, we calculated the Pearson correlation and choose the significant correlation pairs ($P < 0.05$) with which to construct the network.

Genomic Regions Enrichment of Annotations Tool (GREAT)

GREAT assigns biological meaning to a set of non-coding genomic regions by analyzing the annotations of the nearby genes. Thus, it is particularly useful in studying cis functions of sets of non-coding genomic

regions. GREAT analyses were performed by chromosome location of the differentially expressed lncRNAs and the website (<http://bejerano.stanford.edu/great/public/html/>) [19].

Results

Baseline of microarray data

The Gene Expression Omnibus (GEO) database was searched to identify available dataset. After careful searching and validation, the GSE38396 dataset was selected and analyzed [17]. Data analysis pipeline was shown in Figure 1. Raw data of 8 samples (4 diabetic and 4 non-diabetic lymphatic endothelial cell samples, sample ID: GSM941220-GSM941228) were downloaded from the GEO database. Primary quality of the 8 microarrays was assessed by normalized unscaled standard error (NUSE) algorithm and we found that most boxplots of NUSE values were centered around 1, indicating high quality of the microarray data, except for GSM941227 (Fig. 1A). In addition, Relative Log Expression (RLE) analysis also suggests that the GSM941227 sample varied greatly from the other samples. Thus, GSM941227 was excluded from analysis, and 7 samples (4 type 2 diabetes and 3 non-diabetes) were analyzed.

After annotation and statistical analyses, 110 differentially expressed lncRNAs (31 down-regulated and 79 up-regulated) between diabetic and non-diabetic lymphatic endothelial cells were found. Full lists of the 110 lncRNAs were shown in Table 1. Among the 110 lncRNAs, PAX8-AS1 was mostly up-regulated and H19 was mostly down-regulated. As

Fig. 1. Analysis of the GSE38396 dataset. The data analysis flow diagram of current study (A); quality of microarrays were assessed by normalized unscaled standard error (NUSE, B) and relative log expression (RLE, C) methods. Heatmap of the 110 differentially expressed lncRNAs were shown (D).

Table 1. Differentially expressed lncRNAs between diabetic and non-diabetic lymphatic endothelial cells. FC: fold change

reported, the lncRNA H19 is closely related with diabetes and glucose metabolism. [20-22] Several known lncRNAs were found, such as GAS5, UCA1, CRNDE, GAS5, LINC00312.

Co-expression Network

It is proposed that genes with the same biological function or regulated the same pathway way have similar expression patterns. Thus, co-expression network may provide information about function of lncRNAs and co-expression network has been used to predict lncRNA function by many researchers. We built lncRNA-mRNA co-expression network with statistically differentially expressed lncRNA and mRNA (Fig. 2A). Among the network, we found 4 lncRNAs ENSG00000245694 (CRNDE), NR_027012 (LINC00293), NR_015369 (NR2F1-AS1), and ENSG00000230844 (ZNF674-AS1) had highest degrees. Then, sub-networks of the 4 lncRNAs were extracted and the 4 lncRNAs were co-expressed with distinctive gene sets (Fig. 2B).

Genomic Regions Enrichment of Annotations Tool (GREAT) analysis

Recent researches have characterized function of many lncRNAs, however the function of most lncRNAs were still unknown. To predict function of the differentially expressed lncRNAs between diabetic and non-diabetic lymphatic endothelial cells, we performed the GREAT analysis based on genomic location of these lncRNAs [19]. As shown, most of these lncRNAs were located nearby transcription start site (Fig.

Accession ID	logFC	Chr	Name	Gene Start	Gene End	Strand
NR_024582	-0.36	X		73944328	74070408	1
NR_026956	-0.69	19		19756371	19776413	-1
NR_023925	0.45	18		1268311	1359629	-1
NR_026646	0.35	15		24558152	24587779	1
NR_120317	0.45	15		80195484	80252213	-1
NR_002717	0.56	13		70107213	70139753	1
NR_026928	0.46	13		32504506	32509395	-1
NR_028138	-1.01	12		89708955	89711952	1
NR_026932	0.92	10		110868890	110870904	-1
NR_027012	-0.48	8		46840886	46855785	1
NR_015364	0.50	7		26486668	26496367	1
NR_028390	0.33	6		163309985	163324530	-1
NR_027117	0.35	6		35726762	35736947	-1
NR_015369	0.50	5		93541872	93581320	-1
NR_034127	-0.56	5		43065187	43066971	-1
NR_040005	-1.45	3		75672281	75679303	1
NR_024065	-0.86	3		8571782	8574668	1
NR_024606	0.63	2		74502617	74505065	1
NR_024124	0.76	1		116392865	116418622	-1
NR_033998	-0.61	1		94928028	94963270	1
NR_033887	0.45	1		20359801	20428794	-1
NR_002578	0.72	1		173863901	173867987	-1
ENSG00000260386	0.48	1		31500085	31508566	1
ENSG00000241732	-2.03	N/A		N/A	N/A	N/A
NR_015368	1.08	N/A		N/A	N/A	N/A
ENSG00000205662	-1.03	N/A		N/A	N/A	N/A
ENSG00000223482	0.62	N/A		N/A	N/A	N/A
ENSG00000246025	0.41	N/A		N/A	N/A	N/A
ENSG00000224619	0.40	N/A		N/A	N/A	N/A
ENSG00000245443	0.58	N/A		N/A	N/A	N/A
ENSG00000247445	0.87	N/A		N/A	N/A	N/A
ENSG00000233165	-0.51	N/A		N/A	N/A	N/A
ENSG00000237697	-1.01	N/A		N/A	N/A	N/A
ENSG00000226906	0.52	N/A		N/A	N/A	N/A
ENSG00000215560	0.41	Y		22296798	22298876	-1
ENSG00000230844	-0.50	X		46545493	46548408	1
ENSG00000234405	0.40	X		103497523	103500317	1
ENSG00000228839	-0.54	22		31292499	31338021	1
ENSG00000234883	1.02	21		25561909	25575168	1
ENSG00000237282	0.55	20		18379049	18381484	1
ENSG00000213753	-0.64	19		58573503	58599801	1
ENSG00000214049	0.42	19		15828961	15836320	1
ENSG00000268658	0.44	19		21483374	21503238	1
ENSG00000227274	0.44	17		12671862	12706135	-1
ENSG00000179859	-0.85	17		7913324	7915953	-1
ENSG00000249383	0.36	17		51312609	51335165	-1
ENSG00000245694	-1.01	16		54918863	54929189	-1
ENSG00000245694	-1.43	16		54918863	54929189	-1

continued

3A). In addition, several transcription factors (TFs) that are critical for the pathogenesis of diabetes, like MAF, FOX3 were enriched among these genomic regions (Fig. 3) [23, 24]. The enrichment indicated that these TFs might bind to these regions and promote expression of lncRNAs located in these regions. In addition, GREAT analysis also showed that many biological processes were enriched and some were closely related to diabetes, such as “positive regulation of insulin receptor signaling pathway”, “regulation of glycogen biosynthetic process”, and “response to insulin stimulus” (Fig. 3). Enrichment of these biological processes indicated that in lymphatic endothelial cells lncRNA may be involved in the response to insulin and pathological processes of diabetes.

Discussion

lncRNA are a kind of RNA molecules that are larger than 200nt and without protein-coding capacity. Due to advances of high-throughput technology like microarray and the next-generation RNA sequencing (RNA-seq), the functional roles of lncRNA have been discovered [25, 26]. The expression profiles of lncRNA have been characterized in many diseases by microarray and RNA-seq. recently, researchers have demonstrated that annotation of published microarray data is a feasible and reliable method to profile lncRNA expression [27, 28]. In the current study, we re-annotate the HG U133Plus2.0 microarrays and analyzed lncRNA expression profile of lymphatic endothelial cells in diabetic and non-diabetic patients. We found many lncRNAs were differentially expressed in response to type

Accession ID	logFC	Chr Name	Gene Start	Gene End	Strand
ENSG00000247324	0.49	16	71462278	71465941	1
ENSG00000245750	0.42	15	69463026	69571440	1
ENSG00000251533	0.47	14	103187221	103189028	-1
ENSG00000233208	0.41	14	90455230	90458905	1
ENSG00000226722	0.40	13	21872770	21878160	-1
ENSG00000236036	0.36	13	35697524	35699256	1
ENSG00000261057	0.37	13	102589372	102589813	-1
ENSG00000130600	-2.21	11	1995163	2001470	-1
ENSG00000130600	-1.92	11	1995163	2001470	-1
ENSG00000226900	0.46	10	132511626	132518191	-1
ENSG00000225768	0.40	10	104474939	104480274	-1
ENSG00000224596	0.43	10	78943328	79067895	-1
ENSG00000235387	1.37	9	35909483	35911686	1
ENSG00000227388	-0.51	9	35772163	35790432	1
ENSG00000227619	0.41	9	129575409	129584556	1
ENSG00000235659	0.40	9	61865755	61866965	-1
ENSG00000254129	0.43	8	29110573	29140729	1
ENSG00000226869	0.57	7	104738597	104804107	-1
ENSG00000179406	0.70	7	66376044	66401338	-1
ENSG00000228775	0.68	7	141704338	141738346	-1
ENSG00000188185	0.43	7	39733632	39793092	1
ENSG00000188185	0.80	7	39733632	39793092	1
ENSG00000238033	0.35	7	22649960	22665533	-1
ENSG00000244158	0.92	6	116460739	116463692	-1
ENSG00000230910	0.38	6	68629962	68635027	-1
ENSG00000226249	0.40	6	147741434	147743324	-1
ENSG00000246214	0.37	5	16615926	16629969	1
ENSG00000253955	0.34	5	173579643	173585068	1
ENSG00000251603	-0.66	4	151667224	151670502	1
ENSG00000249001	0.43	4	87568035	87733956	-1
ENSG00000248339	0.38	4	171289475	171298267	-1
ENSG00000240024	1.17	3	183447608	183456013	1
ENSG00000239508	0.55	3	155449184	155457753	1
ENSG00000171658	-0.43	3	185959943	185980872	1
ENSG00000244161	0.37	3	58162547	58170636	-1
ENSG00000239513	0.39	3	137771949	137780878	1
ENSG00000235947	-0.97	3	4749192	4751590	-1
ENSG00000242516	-1.96	3	75672391	75679303	1
ENSG00000241163	-0.35	3	72035300	72279503	-1
ENSG00000226383	-0.58	2	156011617	156024613	-1
ENSG00000189223	1.35	2	113211522	113276581	1
ENSG00000229494	0.51	2	78088730	78127806	1
ENSG00000189223	1.39	2	113211522	113276581	1
ENSG00000228203	0.83	2	6912277	6918709	-1
ENSG00000178836	0.43	2	233754305	233755345	-1
ENSG00000230002	-0.63	2	73456764	73459484	1
ENSG00000203643	0.39	2	11721619	11724222	-1
ENSG00000233729	0.35	2	132915557	132931494	1
ENSG00000237638	0.37	2	64901840	64905137	-1
ENSG00000232931	0.40	2	95807118	95816215	-1
ENSG00000224699	0.74	1	110347116	110443817	1

continued

Accession ID	logFC	Chr Name	Gene Start	Gene End	Strand
ENSG00000224702	0.46	1	165210627	165213090	1
ENSG00000224818	-0.59	1	201464383	201465146	-1
ENSG00000237463	0.38	1	165476841	165582155	-1
ENSG00000237058	0.49	1	2632568	2636620	1
ENSG00000203288	0.31	1	151790804	151794402	1
ENSG00000233184	0.87	1	101025878	101087268	1
ENSG00000186056	-0.75	1	30718504	30726827	1
ENSG00000244020	-1.39	1	237004103	237004418	-1
ENSG00000233410	0.38	1	199006040	199021037	-1
ENSG00000231814	0.33	1	217892900	217920804	1
ENSG00000228127	0.45	1	115919350	115931561	1

Fig. 2. Co-expression between differentially expressed lncRNAs and mRNAs (A); subnetwork of ENSG00000245694 (CRNDE), NR_027012 (LINC00293), NR_015369 (NR2F1-AS1), and ENSG00000230844 (ZNF674-AS1) (B).

2 diabetes and functional analyses suggested these lncRNAs were involved in response to insulin stimulus.

Fig. 3. GREAT analysis showed that most lncRNAs were located nearby transcription factors (A). Many insulin- and glucose metabolism related biological processes were enriched by differentially expressed lncRNAs (B). Many transcription factors were enriched in the genomic regions of lncRNAs, like MAF and TCF3 (C).

Type 2 diabetes often leads to inflammation and ulcer [29]. The vascular endothelial cells and lymphatic endothelial cells are exposed to hyperglycemia and dysfunction of endothelial cells is critical for microvascular complications of type 2 diabetes [8]. Previous studies have proved that lncRNAs are involved in the function of vascular endothelial cells and lncRNA expression changes in response to stimulus. For example, Tan Y showed that lncRNA MALAT1 MALAT1 could protect the endothelium from ox-LDL-induced endothelial dysfunction by upregulating CXCR2 [30]. In this study, we found many lncRNAs were differentially expressed in diabetic lymphatic endothelial cells, including several well-known lncRNAs, like CRNDE, H19, and GAS5. H19 was the most down-regulated lncRNA and it has been reported H19 is closely related with diabetes [20]. In this study, we found lncRNA and mRNA were co-expressed in lymphatic endothelial cells and these lncRNAs are highly possible involved in the dysfunction of lymphatic endothelial cells. Additionally, co-expressed network indicated 4 lncRNAs have highest degrees and are the center of network, suggesting they may play central roles. Further experiments are needed to clarify biological functions of these lncRNAs.

To probe the biological function of these differentially expressed lncRNA, GREAT analysis was performed. GREAT analysis refers function of non-coding RNAs based on *cis* regulation [19]. GREAT showed these lncRNAs were enriched in the biological function related with type 2 diabetes, like regulation of insulin pathways and response to insulin stimulus. MAF and TCF3 are critical regulators in type2 diabetes and many MAF-regulated genes are involved in the pathogenesis of type 2 diabetes. GREAT analysis showed MAF and TCF3 were significantly enriched in these genomic regions, indicating these lncRNAs may

be regulated by these TCs. On the other hand, further experiments are needed to validate bioinformatics prediction.

In the current study, we analyzed lncRNA expression of lymphatic endothelial cells in diabetic and non-diabetic patients and this study provides insights into the important roles of lncRNA in lymphatic endothelial cells.

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Disclosure Statement

The authors declare no conflict of interest.

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