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Original Article

Long no-coding RNAs and mRNAs expression profile in human colorectal cancer: analysis based on the B7-H4 regulation

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Abstract: B7-H4 is now recognized as a kind of co-stimulatory proteins that can interact with the ligands, and it has been extensively proven to have the ability in not only inhibiting T cell proliferation but also inducing cell cycle arrest and at the same time decreasing the secretion of cytokines, exhibiting an independent prognostic indicator for various cancers. However, studies concerning how to regulate long non-coding RNAs (lncRNAs) and mRNAs in the human colon cancer by the B7-H4 are still seldom noticed by previous works. Here, we report on using the RNA-sequencing (RNA-seq) and bioinformatic analysis to profile differential expression genes in SW1116 with or without over-expressed B7-H4. A total of 167 differential genes, including 39 lncRNAs and 128 mRNAs, are identified. Our findings in RNA-seq are validated randomly using qRT-PCR, and results reveal that GO and KEGG analyses play the role in predicting the possible roles of B7-H4 in colon cancer cells, namely, B7-H4 contributes to regulate the protein processing and mRNA surveillance related pathways.

Keywords: B7-H4 expression, colorectal cancer, long non-coding RNAs, RNA-seq

Introduction

V-set domain-containing T-cell activation inhibitor 1 (VTCN1), which is also known as B7-H4, belongs to the B7 superfamily. The family members are co-stimulatory proteins to interact with the ligands like CD28 on T lymphocytes [1]. Up until now, it has been found that B7-H4 can suppress T cell proliferation, resulting in inducing cell cycle arrest and decreasing the secretion of cytokines, such as IL-2, IL-4, IL-10, and IFN-γ [2]. As a result, B7-H4 has acted as a distinct member of B7-family, and it has been extensively suggested for expressing the lung, colon, kidney, skeletal muscle, pancreas, and pancreas [3, 4]. However, the existence of B7-H4 protein in normal cell is very limited [3]. Despite the tightly expressed protein in normal cells, various cancer tissues over xpress B7-H4 protein, and the expression level is found to be related to tumor size, TNM stage, and survival period [5, 6]. One meta-analysis, enrolled 3771 patients with different kinds of cancers, demonstrates B7-H4 is an independent predictive symbol of cancer patients [7].

Over the past decades, the improvement of RNA microarrays and high-throughput sequencing have shown the wide expression of long non-coding RNAs (lncRNAs) in human cells and their crucial roles in various diseases, including cancers [8]. LncRNAs are described as a group of RNAs with more than 200 nucleotides in length, which are not predicted to encode proteins. They have been proven to not only regulate transcription in cis or trans and proteins or RNA molecules but also affect other RNAs processing and organize nuclear domains [9]. However, some transcripts, annotated as putative lncRNAs, are actually translated into proteins [10, 11].

Although there are a lot of research articles concerning the functions of B7-H4 and lncRNAs in colon cancer, how B7-H4 to regulate lncRNAs still remains unknown. Unlike our previous work

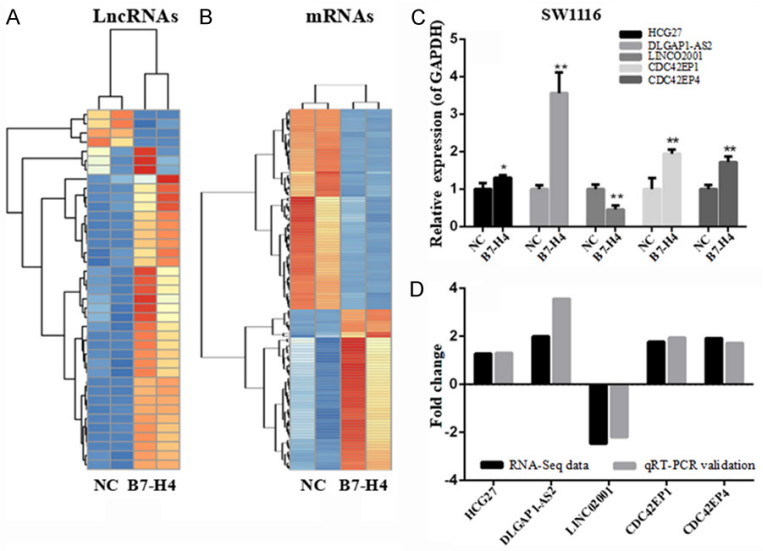

Figure 1. Differential expression of lncRNAs (A) and mRNAs (B) with and without B7-H4; Validation of RNA-seq data by qRT-PCR results for 3 lncRNAs and 2 mRNAs, (C) and (D) are qRT-PCR results and the comparison between qRT-PCR result and RNA-seq data, respectively.

in which we has proved the expression level of B7-H4 in colon cancer tissues can be apparently elevated as compared to the adjacent normal ones and B7-H4 siRNA that can prohibit efficiently proliferation, invasion, and migration of colon cancer cells [12], here we carry out this study to investigate differentially expressed lncRNAs and mRNAs in human colon adenocarcinoma cell SW1116 with or without overexpression of B7-H4, aiming to dig out a deeper mechanisms of B7-H4 regulation in colon cancer.

Materials, analysis methods and experimental detail

Plasmids, cell culture, transfection and lentivirus infection

The plasmid of pLX304-VTCN1 was obtained from Shanghai Jiaotong University School of Medicine, Shanghai, China, and so was the mock-vehicle for negative control. The human embryonic kidney cell HEK-293T and human colon adenocarcinoma cell SW1116 was purchased from American Type Cell Culture (ATCC). All cells were cultured according to culture methods offered by ATCC (www.atcc.org). The transfection was performed by using Lipofectamine 3000 (Invitrigen, CA, USA), when cells grew to 60-80% confluence. Supernatant containing lentivirus was packed in HEK-293T cells. Viral supernatant was added into 60-70% confluence cells for infection. After 48 h, cells were incubated with 5 μg/ml blasticidin (Thermo Fisher Scientific, MA, USA) for selection over 1 week.

RNA library construction and sequencing

Total RNA was drawn out from cells with TRIzol reagent (Invitrigen, CA, USA) as approved protocol. The total RNA was sent to a tech service provider (LC-Bio Tech, Hangzhou, China) in carbon dioxide ice for RNA library construction and sequencing. RNA samples, suffered from quality control (RNA 6000 Nano LabChip Kit, Agilent, CA, USA) and purification (Epicentre Ribo-Zero Gold Kit, Illumina, San Diego, USA), were broken into small pieces and reverse-transcribed to build the cDNA library as recommended protocol (Illumina, San Diego, USA). The sequencing was then performed on Illumina X10 (LC-Bio Tech, Hangzhou, China).

Transcripts assembly

Adapter contaminations were abandoned through Cutadapt and quality of sequence was certified by FastQC [13]. Clean reads were then mapped to the reference genome built with Topaht2 and Bowtie2.

LncRNA identification

Transcripts that overlapped with known mRNAs nad transcripts (shorter than 200 bp) were abandoned. Coding Non-Coding Index (CNCI), Coding Potential Calculator (CPC) were utilized to predict transcripts for coding capability. All transcripts with CNCI score < 0 and CPC score < -1 were discarded. The remaining transcripts were treated as lncRNAs.

Different expression analysis of lncRNAs and mRNAs

String Tie was used to perform expression level for mRNAs and lncRNAs by calculating FPKM [14]. Differential expression of lncRNAs and

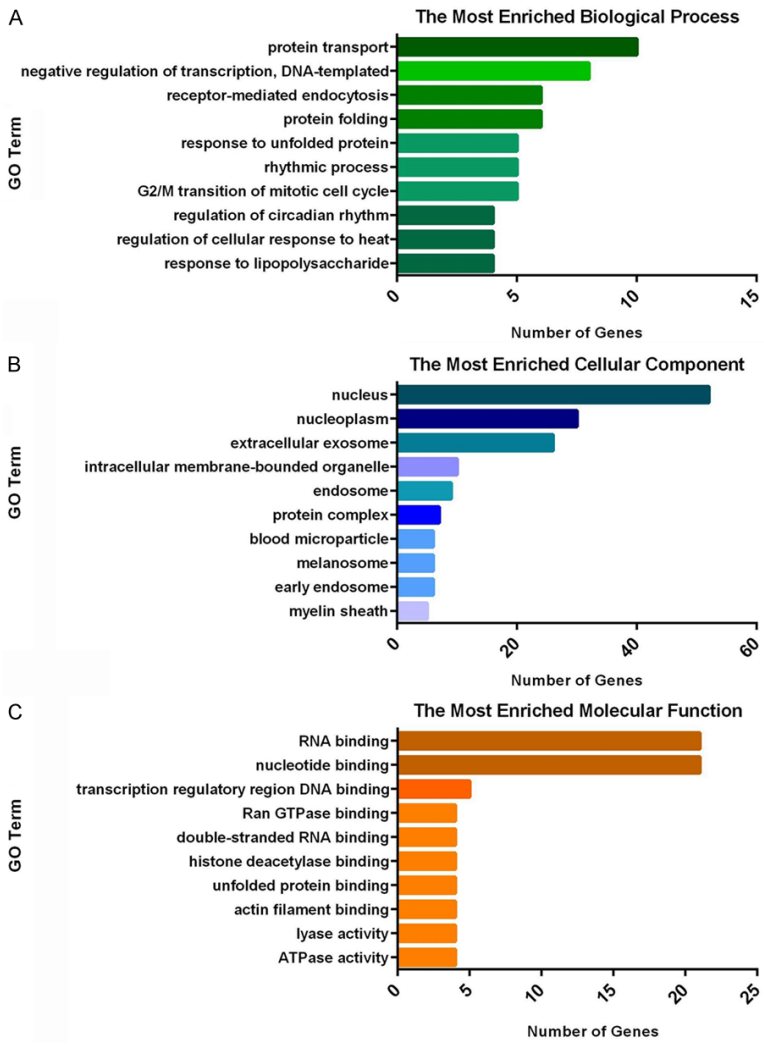

Figure 2. Function analysis of differentially expressed mRNAs (top 10), (A) is the most enriched biological process, (B) is the most enriched cellular component and (C) is the most enriched molecular function.

mRNAs was chosen with absolute value of log2 (fold change, FC) > 1 and with statistical significance (p value < 0.05) by R package [15].

Target gene forecast and functional analysis for lncRNAs

To seek the function of lncRNAs, the *cis*-target and *trans*-target genes of lncRNAs were predicted to investigate probable functions by BLAST2GO [16]. The *trans*-target genes of lncRNAs were predicted through free energy between RNA secondary structures. The mRNAs 100 kb up- and downstream of lncRNAs, on the other way, were presumed to be *cis*-target genes. Significance was expressed as a p value < 0.05.

Quantitative real time-polymerase chain reaction (qRT-PCR)

RNA extraction was carried out as we described above. Reverse transcription was performed by TUREscript 1st Stand cDNA SYNTHESIS Kit (Aidlab, Beijing, China). The quantification and analysis of target mRNAs and lncRNAs were performed using SYBR Green qPCR (ABI, Foster City, CA, USA) with specific primers, while GAPDH as the internal control. The procedure was listed in our study before [17].

Experimental results

Differentially expressed lncRNAs and mRNAs in SW1116 with over-expression of B7-H4

A genome-wide sequencing was performed to identify atypical expression of lncRNAs and mRNAs from duplicated samples of SW1116 with and without over-expression of B7-H4. A total of 39 lncRNAs and 128 mRNAs were chosen to differentially express. Of these, 35 lncRNAs and 71 mRNAs were up-regulated ones in the group over-expressed B7-H4 as compared to negative control (NC) group. In the meanwhile, 4 lncRNAs and 57 mRNAs were down-regulated ones. The differently expressed lncRNAs (A) and mRNAs (B) were shown in **Figure 1**.

Validation of transcription levels of lncRNAs and mRNAs

To verify the RNA-seq data, the expression of lncRNAs and mRNAs in the third pair of cells with and without over-expressed B7-H4 was detected by qRT-PCR. A total of 3 lncRNAs (HCG27, DLGAP1-AS2 and LINC02001) and 2 mRNAs (CDC42EP1 and CDC42EP4) were randomly selected (see **Figure 1C**). The compari-

Table 1. Enriched KEGG pathways (mRNAs)

Pathway ID	Pathway Name	Genes	*p* value
ko03320	PPAR signaling pathway	ANGPTL4; LPL; PCK2; UBC	0.00
ko04141	Protein processing in endoplasmic reticulum	DNAJA1; HSP90AA1; HSPA8; HSPH1; LMAN1; YOD1	0.00
ko04920	Adipocytokine signaling pathway	ADIPOR2; PCK2; SLC2A1	0.01
ko04144	Endocytosis	ARF6; CHMP1B; HSPA8; PIP5KL1; TFRC	0.02
ko00350	Tyrosine metabolism	FAHD1; MAOB	0.03
ko03015	mRNA surveillance pathway	NXT1; PNN; WDR82	0.03
ko04915	Estrogen signaling pathway	HBEGF; HSP90AA1; HSPA8	0.04
ko03022	Basal transcription factors	TAF5; TAF9	0.04

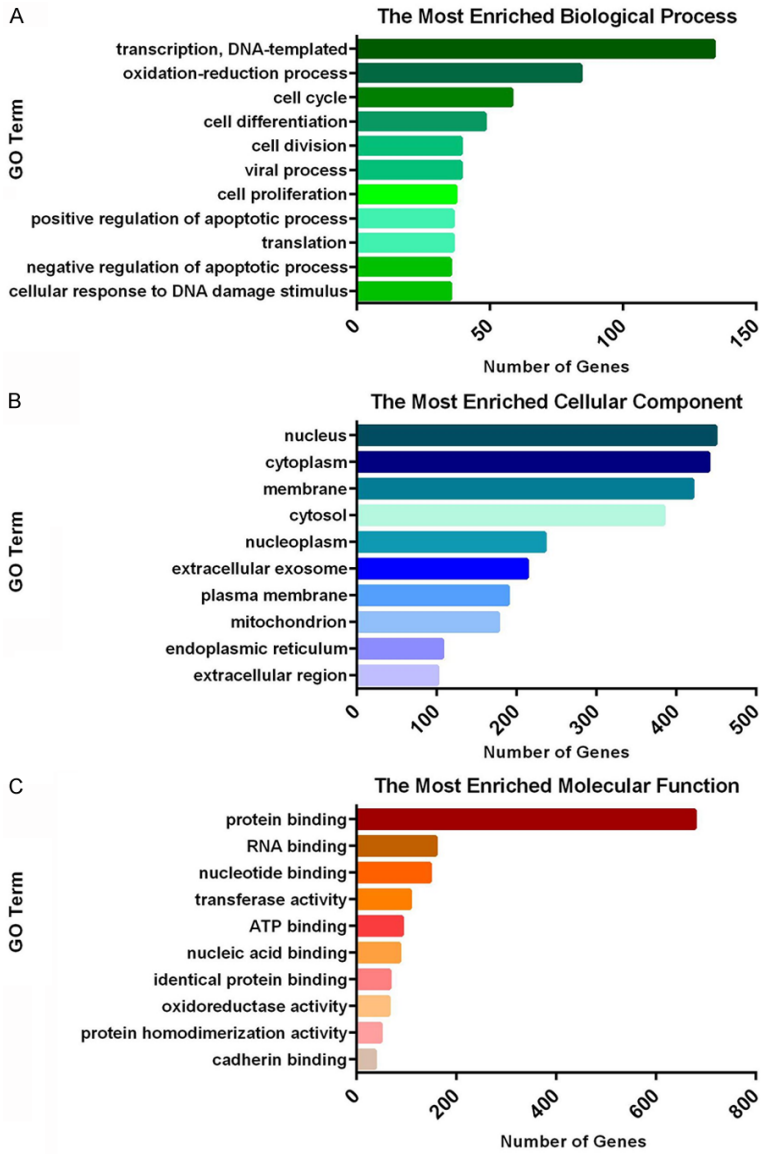

Figure 3. Functional analysis for differentially expressed lncRNAs and mRNAs (top 10), (A) is the most enriched biological process, (B) is the most enriched Cellular Component and (C) is the most enriched molecular function.

son between qRT-PCR and RNA-seq data showed similar trends (see **Figure 1D**). The consistency proved the accuracy and reliability of RNA-seq data.

Functional analysis for differentially expressed mRNAs

Based on the number of differentially expressed mRNAs, enriched biological processes (BPs), cellular components (CCs) and molecular functions (MFs) can be listed, as shown in **Figure 2**. The most represented BPs include various functional, typically in the aspects such as the protein transport, negative regulation of transcription, receptor-mediated endocytosis, protein folding, response to unfolded protein, rhythmic process, G2/M transition of mitotic cell cycle, regulation of circadian rhythm, response of cellular regulation to heat and lipopolysaccharide. The enriched CCs were concentrated mainly on nucleus, nucleoplasm and extracelluar exosome. According to the number of genes that serves for MFs, the top 10 MFs were show as follows: RNA binding, nucleotide binding, transcription regulatory region DNA binding, Ran GTPase binding, double-stranded RNA binding, histone deacetylase binding, unfolded protein binding, actin filament binding, lyase activity and ATPase activity. In this study, 8 KEGG pathways were enriched based on a cutoff criteria of $P < 0.05$

Table 2. Enriched KEGG pathways (lncRNAs and mRNAs)

Pathway ID	Pathway Name	Gene Number	*p* value
ko04141	Protein processing in endoplasmic reticulum	24	0.00
ko03013	RNA transport	23	0.00
ko00190	Oxidative phosphorylation	22	0.00
ko04110	Cell cycle	22	0.00
ko03010	Ribosome	17	0.00
ko04066	HIF-1 signaling pathway	16	0.00
ko03015	mRNA surveillance pathway	16	0.00
ko04922	Glucagon signaling pathway	16	0.00
ko00240	Pyrimidine metabolism	15	0.00
ko04360	Axon guidance	13	0.03

Table 3. Enriched KEGG pathways referred to diseases (lncRNAs and mRNAs)

Pathway ID	Pathway Name	Gene Number	*p* value
ko05010	Alzheimer's disease	26	0.00
ko04932	Non-alcoholic fatty liver disease (NAFLD)	25	0.00
ko05016	Huntington's disease	23	0.00
ko05169	Epstein-Barr virus infection	23	0.01
ko05203	Viral carcinogenesis	22	0.00
ko05012	Parkinson's disease	20	0.00
ko05164	Influenza A	20	0.00
ko05168	Herpes simplex infection	18	0.04
ko05162	Measles	16	0.00
ko05160	Hepatitis C	14	0.01

(**Table 1**). These results indicate us that B7-H4 has been involved in the pathways associated with endocrine system (ko03320, ko04920, ko04915), cellular processes (ko04144), genetic information processing (ko04141, ko03015, ko03022) and metabolism (ko00-350).

LncRNA cis- or trans-target prediction

According to previous results [9], lncRNA regulating gene expression can be broadly classified into the act in cis (*i.e.*, affecting the expression of nearby genes) and trans (*i.e.*, influencing the gene expression or chromatin states in region distant from the transcription site). In this work, the trans-target genes of lncRNAs were predicted through free energy between RNA secondary structures. On the other way, mRNAs 100 kb up- and downstream of lncRNAs were considered as the cis-target genes. The differential lncRNAs were annotated by analyzing the functions of these target mRNAs, and the results have been shown in **Figure 3**. In the figure, the enriched GO terms were shown to be a bit different from the result of solo mRNAs analysis. The most enriched BPs includes transcription, oxidation-reduction process, cell cycle and so on. The most enriched CCs are combined with nucleus, cytoplasm, membrane and cytosol. It is obvious that the genes referred to RNA binding and nucleotide binding are more than other MFs.

More than that, there are also a lot of differences in the enriched KEGG pathways, as listed in **Tables 2**, **3**. The top 10 enriched pathways refer to metabolism (ko00190, ko00-240), development (ko-04360), endocrine system (ko04922), cellular processes (ko04110), genetic information processing (ko04141, ko-03015, ko03013, ko03010) and signal transduction (ko04066). Furthermore, the top 10 diseases enriched by differentially expressed genes are associated with not only cancers (ko05203), but also endocrine and metabolic disease (ko04932), neurodegenerative diseases (ko05010, ko05012, ko05016) and infectious diseases (ko05162, ko05164, ko05160, ko05168, ko05169).

Survival analysis for lncRNAs

In order to verify whether the differential lncRNAs influence colon cancer patients' survival, we used GEPIA18 (Gene Expression Profiling Interactive Analysis, http://gepia.cancer-pku.cn/), a web tool which is based on TCGA and GTEx data to analyze. Of 39 lncRNAs, DNAJC9-AS1, HCG27 and RP11-339B21.13 are showed to be independently factors to predict patients' overall survival (OS) by using quartile cutoff for high or low expression division ($p < 0.05$). These 3 lncRNAs were up-regu-

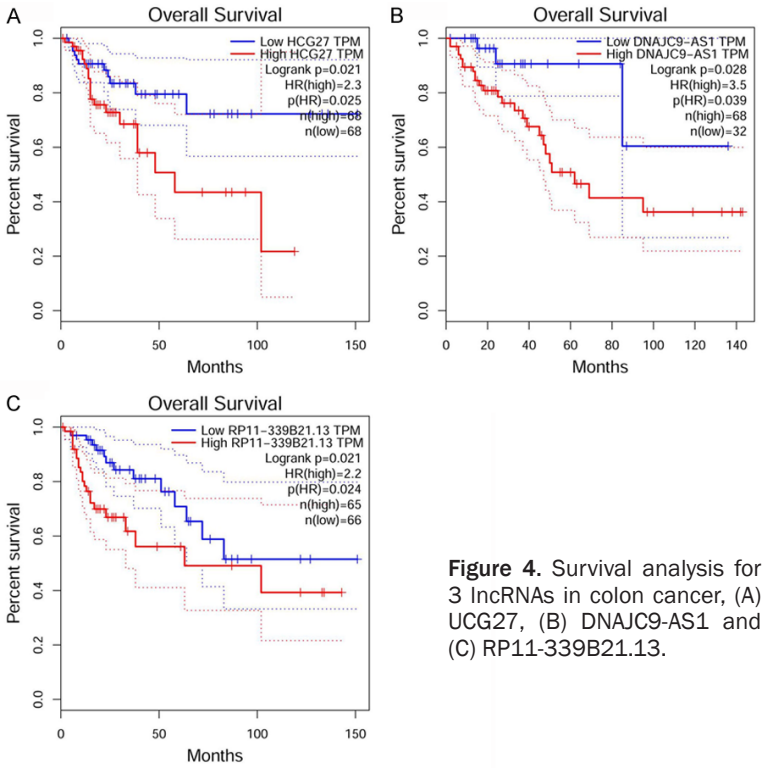

Figure 4. Survival analysis for 3 lncRNAs in colon cancer, (A) UCG27, (B) DNAJC9-AS1 and (C) RP11-339B21.13.

lated markedly by over-expressed B7-H4, and furthermore, patients in the 3 high-expression groups had dramatically shorter mean OS than those in the low-expression ones (see **Figure 4**). Further research on the correlation between B7-H4 and the 3 lncRNAs in colon cancer will be valuable.

Discussion

The incidence and mortality rate of colon cancer has kept growing in the past few years. The latest five-year survival rate of colon cancer patients in China has been up to 57.6% [19]. In recent years, the immunotherapeutic approaches, such as cytotoxic T-lymphocyte-associated protein 4 (anti-CTLA-4), and programmed death-ligand 1 (anti-PD-1), have been considered as an efficient effect for cancer treatment, but have only served for a subgroup of patients [1, 20]. The expression of B7-H4 is increased noticeably in various cancers, making it being a promising alternative target for cancer research. To our best knowledge, the present study is the first one to report differential expression of lncRNAs and mRNAs in colon cancer cells with or without over-expressed B7-H4.167 differential genes, including 39 lncRNAs and 128 mRNAs, have been chosen between 2 over-expressed B7-H4 samples and 2 negative control ones. The results for qRT-PCR in another pair of cell samples validated the findings in RNA-seq.

Depending upon RNA-seq data, IMP3, as a RNA-binding protein, is the most up-regulated mRNA in over-expressed B7-H4 group. Its over-expression was observed in various cancers, in which it contributed to poor prognosis [21, 22]. On the other hand, CHMP1B, a peripherally associated component of endosomal sorting complexes required for transport complex III (ESCRT-III), is the most down-regulated one. It involves multivesicular body assembly, protein transport. It has been demonstrated that it can be used to regulate the autophagic turnover of plastid constituents in plants [23, 24]. Except for the only one over-expressed CHMP1B in esophageal squamous cell carcinoma tissues [25], there is still no previous works concerning CHMP1B and cancers. The lncRNAs (*e.g.*, DLGAP1-AS2 and LINC02001) which can be regulated largely by B7-H4 have been validated by some novel lncRNAs that can get only gene IDs. The functions of these lncRNAs, however, still remain unclear, but we could predict their possible roles via the target mRNAs. Obviously, the result of functional analysis for differential mRNAs alone is quite different from the one for mRNAs combined with lncRNAs. As the former one is analyzed at gene level by the differential mRNAs only, the latter one is analyzed by the target mRNAs interacting with differential lncRNAs in transcriptional level.

Protein processing in endoplasmic reticulum (ER) is one of the most enriched KEGG pathways for both mRNA solo analysis and lncRNAs combining with mRNA analysis. It implies that B7-H4 may unsettle ER protein folding capacity and led to so-called “ER stress”, which is uncovered to impede the protection of anti-cancer immunity yet again [26].

The mRNA surveillance pathway which is used to ensure quality and fidelity of messenger RNAs (mRNAs) is another one enriched in both analyses. The surveillance is achieved by marking abnormal mRNAs for endogenous nucleases destruction. Nevertheless, cancers are discovered to exploit this process to degrade some key tumor-suppressor genes such as p53 [27].

As B7-H4 is a negative factor in T lymphocytes response, the differential genes is associated with infectious diseases such as measles, Epstein-Barr virus, herpes simplex infection, influenza A and hepatitis C as expected. Interestingly, we also get a fair number of neurodegenerative diseases in the enriched KEGG pathway list. These diseases including Alzheimer's disease, Parkinson's disease and Huntington's disease have similar amyloid origins caused by accumulation of misfolded proteins [28]. According to the most recently-reported work [29], depletion of $Foxp^{3+}$ regulatory T cells (*i.e.*, Tregs) can help to mitigate Alzheimer's disease pathology, the presence of which is correlated positively with B7-H4 expression. Tregs also play an important role in regulating inflammatory processes in liver disease [30]. It gives the explanation for why there are so many differential genes involved in non-alcoholic fatty liver disease (NAFLD).

Additionally, here we also explore the prognostic value of differential lncRNAs based on TCGA and GTEx data in colon cancer. Only 3 lncRNAs (DNAJC9-AS1, HCG27 and RP11-339B21.13) are showed to be independently prognostic indicators, while others are excluded because they had not been validated yet.

Conclusion

In this work, we comprehensively analyzed lncRNAs and mRNAs regulated by B7-H4 for the first time, in which the RNA-sequencing (RNA-seq) and bioinformatic analysis was used to profile differential expression genes in SW1116 with or without over-expressed B7-H4. Our findings showed that B7-H4 contributes to regulate the protein processing and mRNA surveillance related pathways. This work we exhibited here enables us to understand better not only the role of B7-H4 in colon cancer in immune aspect, but also the protein processing and, in the meanwhile, the mRNA surveillance related aspect.

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Disclosure of conflict of interest

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