The Association Between lncRNA LRRC75A-AS1 and The Clinical Characteristics in Neuroblastoma

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Abbreviations: NB: Neuroblastoma; lncRNAs: Long Noncoding RNAs; LDH: Lactate Dehydrogenase; NSE: Neuron-Specific Enolase; ORFs: Open Reading Frames; uFH: Unfavorable Histology; FH: Favorable Histology; NA: Not Amplified; NM: Not Metastasis; PD: Progressive Disease; VMA: Vanilla Mandelic; SNHG29: Small Nucleolar RNA Host Gene 29; TJ: Tight Junction; CRC: Colorectal Carcinoma

ABSTRACT

Background: Neuroblastoma is the highest mortality rate extracranial solid tumor in childhood. Accumulating evidence indicated that long noncoding RNAs (lncRNAs) are widely expressed in neuroblastoma, and playing an important role in the development and progression.

Methods: RNA sequencing was conducted to identify differentially expressed lncRNAs in four III phase and four IV phase tumor tissues of neuroblastoma. RT-qPCR was carried out to validate the result of sequencing. Clinical information was reviewed to analyze the relationship between lncRNA and clinical characteristics. The public database R2 was used to analyze prognosis.

Result: Differentially expressed lncRNAs were identified. LRRC75A-AS1 was the overexpressed lncRNA in IV phase patients. RT-qPCR was conducted in tumor tissues, confirming the tendency with sequencing. And higher expression of LRRC75A-AS1 was associated with N-MYC (p < 0.001), advanced stage (p = 0.029), Risk group (p = 0.027). Furthermore, LRRC75A-AS1 was correlated with Shimada classification (p = 0.046), LDH level (r = 0.390, p = 0.003), D-Dimer level (r = 0.338, p = 0.012) , and NSE level (r = 0.284, p = 0.05). The neuroblastoma dataset shows that patients with overexpressed LRRC75A-AS1 have a worse prognosis than down-expressed.

Conclusion: LRRC75A-AS1 is associated with clinical characteristics of neuroblastoma and may function as a prognostic predictor or a therapeutic target.

Introduction

Neuroblastoma (NB) is a sympathetic embryonic tumor originating from the neural crest of the embryonic sympathetic nervous system, it is the most common extracranial solid tumor in children which accounts for 7-10% of all childhood cancer mortality [1-5]. In order to take tailored treatment approaches for neuroblastoma, pediatric cooperative groups introduce risk factors including clinical stage, age, histologic category, grade of tumor differentiation, MYCN status, DNA ploidy, and 11q exception [6,7]. High-risk neuroblastoma patients often have unfavorable outcomes, with the 5-year overall survival rate less than 50% [6]. The application of genetic difference analysis promote accurate stratification has attracted widespread attention. Therefore, it is of great practical significance and theoretical value to explore effective drug targets and better biomarkers for advanced neuroblastoma.

Long noncoding RNAs (lncRNAs) refer to endogenous RNAs that are longer than 200 nucleotides and lack of specific complete open reading frames (ORFs) and the function of protein-coding [8,9].

Thus they were once considered a part of transcriptional noise, but now have been proved as potential key regulators of promoting or maintaining tumorigenesis and the development of cancer, having clinical potential as prognostic biomarkers for targeted therapeutics and interventions in various cancers [4,10]. Several lines of evidence have shown that lncRNAs have been implicated in initiation and progression of neuroblastoma [5,6], and lncRNA-based prognostic biomarkers have been proposed for tumor stratification and predicting survival. Therefore, deep investigating of the roles and mechanisms of lncRNAs in tumorigenesis provides promises in developing new biomarkers and molecular-targeted therapy. Our research aims to identify lncRNA-based biomarkers.
that could be used for prognosis prediction and treatment. The purpose of this study was to explore the relationship between IncRNAs and clinicopathological parameters in neuroblastoma patients, to further explore the IncRNAs that lead to the invasion and metastasis of neuroblastoma. In the first, we conducted RNA-sequencing to identify differentially expressed IncRNAs in 4 III stage and 4 IV stage patients' tumor tissues of neuroblastoma, and we identified a IncRNA named LRRC75A-AS1 was upregulated IncRNAs in IV stage neuroblastomas, further to explore the relationship between LRRC75A-AS1 and clinical characteristics, RT-qPCR was carried out to detect 57 cases of neuroblastoma.

Materials and Methods

Patient

A total of 57 cases of fresh primary tumor tissues with pathologically diagnosed neuroblastoma were collected in this study approved by the ethics committee of the Children's Hospital of Chongqing Medical University from August 2014 to May 2019. And subjects (or their parents or guardians) have given their written informed consent.

The Inclusion Criteria

a) Pathologically diagnosed NB.

b) Primary tumor without any treatment.

c) Written informed consent was obtained from the guardians.

The Exclusion Criteria

a) History of other malignant disease.

b) Recurrent or treated disease.

c) The quality of tissues was unqualified.

Clinical features of these patients at diagnosis including age, gender, tumor size, INSS stage, risk group, MYCN status, tumor biomarkers, and metastasis were retrospectively collected. All fresh tissue specimens were preserved in −80°C until use. R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl) was used to investigate the relationship between IncRNA expression and overall survival with neuroblastoma patients.

Expression Profile Analysis of RAN-Sequencing

The RAN-sequencing was employed to identify neuroblastoma-related RNAs. After hybridization and washing with samples, 8 samples of extracted RNA of neuroblastoma tumor tissues (4 III stage neuroblastoma and 4 IV stage neuroblastoma) were analyzed.

RNA Extraction and Real Time qRT-PCR Analysis

Total RNA of specimens was extracted using RNA extraction reagent kit (Bio Teke). RNA concentration and purity were measured by NonoDrop (Thermo Scientific). cDNA was reverse transcribed with the Prime Script RT reagent Kit ((Takara Biotechnology Co., Ltd, China) from 1000 ng of total RNA. The real-time qPCR analyses were carried out using SYBR GREEN Premix ExTaq kit (Danfeng, China) by CFX96 Cycler System. Relative RNA expression was computed by 2^ΔΔCt method with normalization to human β-actin. The primers for LRRC75A-AS1 are: F 5′-AGCTCACGCAACCTGCTA-3′ and R 5′-AGCTAGGCAAGGACGATC-3′, and the primers for β-actin are: 5′-CCTGGCACCCAGCACAAT-3′ and R 5′-GGCCGGACTCGTCATAC-3′.

Statistical Analysis

Statistical analyses were conducted by SPSS 23.0 (IBM Corporation, Armonk, NY, USA). Graphical depiction of data was generated by GraphPad Prism v5.0. (GraphPad Software, Inc., La Jolla, CA). In the statistical analysis, a two-sided p value <= 0.05 was considered statistically significant. Differentially expressed IncRNAs were identified through fold change as well as P value calculated with t-test. The threshold set for up- and downregulated genes was a fold change >= 2.0 and a p value <= 0.05. For qualitative data, the χ2 test or Fisher exact test was used to evaluate the significance between groups. For quantitative data, Kruskal-Wallis test was used to analyze the significance between individual groups. The correlations were analyzed by Spearman correlation analysis. The prognostic relationship was evaluated using Kaplan-Meier.

Result

Screening for Differentially Expressed IncRNAs

To investigate narrow metastasis-related RNA expression profile in neuroblastoma tissues, we analyzed 8 tissue samples of neuroblastoma (4 III stage and 4 IV stage patients) by using the RNA-sequencing. The pathological characteristics of the 8 patients are listed in Table 1. With the threshold set for up- and downregulated genes of a fold change >= 2.0 and a p value <= 0.05, 1043 differentially expressed IncRNAs were identified between III and IV stage tumor samples, including 458 upregulated IncRNAs and 585 downregulated IncRNAs. Among them, we found that LRRC75A-AS1 was the overexpressed IncRNA in IV stage patients with the fold change of 3.19 (p = 0.02, (Figure 1). To determine the tendency of sequence, RT-qPCR was carried out to measure LRRC75A-AS1 expression level of 57 neuroblastoma tumor tissues, including the 8 patients' tumor tissues for sequencing. The relative expression of LRRC75A-AS1 ranged from 0.03 to 5.62, with the median value of 0.39. Compared to the III stage tissues, expression of LRRC75A-AS1 was higher in IV stage tissues, however it's not statistically significant (p = 0.152). But compared to the early stage tissues, expression of LRRC75A-AS1 was higher in advanced stage tissues (p = 0.029) (Figure 2A). LRRC75A-AS1 was higher in high-risk than intermediate-risk and low-risk neuroblastoma (p = 0.027) (Figure 2B). LRRC75A-AS1 was higher in N-MYC amplified disease than N-MYC Not-amplified disease (p < 0.001) (Figure 2C). The results suggesting that IncRNA LRRC75A-AS1 may play a significant role in the pathogenesis and development of neuroblastoma.
Figure 1: The volcano plot showed differentially expressed lncRNAs between III stage and IV stage neuroblastoma tumor samples.

Figure 2: The correlation between expression of LRRC75A-AS1 and Stage, Risk classification, N-MYC.
(A) Relative expression of LRRC75A-AS1 in early (I, II, IVs) and advanced (III, IV) stage disease.
(B) Relative expression of LRRC75A-AS1 in low and intermediate (low + inter) and high-risk neuroblastoma.
(C) Relative expression of LRRC75A-AS1 in N-MYC(MYCN) not amplified, and N-MYC amplified.
Table 1: Clinicopathological parameters of 8 patients whose tumors were used for RNA-sequencing.

| Patients | Age  | Gender | INSS Stage | Risk Group | Histologic Classification | MYCN Status | Marrow Metastasis | Outcome  |
|----------|------|--------|------------|------------|---------------------------|-------------|-------------------|----------|
| A1       | 6 yrs| Female | IV         | High       | uFH                      | NA          | Metastasis        | Alive    |
| A2       | 5 yrs| Female | IV         | High       | uFH                      | NA          | Metastasis        | -        |
| A3       | 4 yrs| Female | IV         | High       | uFH                      | NA          | Metastasis        | Alive    |
| A4       | 6 yrs| Male   | IV         | High       | uFH                      | NA          | Metastasis        | Alive    |
| B1       | 4 yrs| Female | III        | High       | uFH                      | NA          | NM                | PD       |
| B2       | 1 yrs| Male   | III        | High       | uFH                      | NA          | NM                | Alive    |
| B3       | 3 yrs| Female | III        | High       | uFH                      | NA          | NM                | Alive    |
| B4       | 12 yrs| Male   | III        | High       | uFH                      | NA          | NM                | Alive    |

Note: INSS: International Neuroblastoma Staging System; Inter: Intermediate; uFH: Unfavorable Histology; NA: Not Amplified; NM: Not Metastasis; PD: Progressive Disease

Correlations Between the Expression Level of LRRC75A-AS1 and Clinical Characteristics

The 57 neuroblastoma patients were divided into two groups (high or low) based on the median value of LRRC75A-AS1 expression level (Table 2). We found that high LRRC75A-AS1 expression level in tumor tissues was associated with advanced INSS stage (p = 0.039), risk group (p = 0.004), N-MYC status (p = 0.001), shimada classification (p = 0.046). Moreover, we identified that LRRC75A-AS1 expression was correlated with serum lactate dehydrogenase (LDH) level (r = 0.390, p = 0.003) (Figure 3A), D-Dimer level (r = 0.338, p = 0.012) (Figure 3B), and serum neuron-specific enolase (NSE) level (r = 0.284, p = 0.05) (Figure 3C), but has no correlation with ki-67 level (p = 0.163), Vanilla mandelic acid (VMA) level (p = 0.073), and tumor size (p = 0.515).

Figure 3: The correlation between expression of LRRC75A-AS1.
(A) LDH
(B) D-Dimer
(C) NSE
Table 2: Relationships between the expression of LRRC75A-AS1 and clinicopathological parameters in neuroblastoma

| Factors               | LRRC75A-AS1 Expression | x2   | P       |
|-----------------------|------------------------|------|---------|
|                       | High (n=29)            | Low (n=28) |       |
| Age                   |                        |      |         |
| ≥1 year               | 23                     | 17   | 2.354   | 0.155  |
| < 1 year              | 6                      | 11   |         |
| Gender                |                        |      |         |
| Male                  | 19                     | 16   | 0.422   | 0.592  |
| Female                | 10                     | 12   |         |
| INSS Stage            |                        |      |         |
| I + II + IVS          | 1                      | 6    | 4.275   | 0.039  |
| III + IV             | 28                     | 22   |         |
| Risk group            |                        |      |         |
| Low + Intermediate    | 2                      | 11   | 8.489   | 0.004  |
| High                  | 27                     | 17   |         |
| Shimada Classification|                        |      |         |
| uFH                   | 25                     | 15   | 3.987   | 0.046  |
| FH                    | 4                      | 9    |         |
| Missing               | 0                      | 4    |         |
| N-MYC Status          |                        |      |         |
| Amplified             | 12                     | 1    | 11.924  | 0.001  |
| NA                    | 14                     | 24   |         |
| Missing               | 3                      | 3    |         |
| Marrow Status         |                        |      |         |
| Metastasis            | 17                     | 10   | 2.998   | 0.083  |
| NM                    | 12                     | 18   |         |

Note: INSS: International Neuroblastoma Staging System; uFH: Unfavorable Histology; FH: Favorable Histology; NA: Not Amplified; NM: Not Metastasis

Relations Between Expression Level of LRRC75A-AS1 and Prognosis in Neuroblastoma Patients

Owing to the small sample size and limited follow-up time of partial patients, we cannot analyze the correlation between overall survival and LRRC75A-AS1 in our patients. Thence, we see progressive disease or died as outcome indicator to investigate the relationship of LRRC75A-AS1 expression level and prognosis with neuroblastoma patients. The results show that high expression of LRRC75A-AS1 was correlated with poor prognosis ($\chi^2=5.662$, $p = 0.017$) (Table 3). Further to investigate the relationship of LRRC75A-AS1 expression and overall survival in neuroblastoma patients, R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl) was used to analyze the relationship (Figure 4). Kaplan–Meier analysis demonstrated elevated LRRC75A-AS1 expression levels were associated with poor prognosis, whereas low expression of LRRC75A-AS1 was associated with favorable outcome in the Versteeg dataset consisting of a cohort of 88 neuroblastoma patients ($n = 88$, log-rank: $p = 0.027$). To sum up, our analysis of LRRC75A-AS1 with clinical features and microarray dataset indicated that LRRC75A-AS1 was a novel prognostic marker in neuroblastoma.

Table 3: The relationship of LRRC75A-AS1 expression and overall survival.

| Outcome   | LRRC75A | $\chi^2$ | P      |
|-----------|---------|----------|--------|
|           | High    | Low      |        |
| PD/Died   | 14      | 6        | 5.662  | 0.017  |
| Live      | 9       | 17       |        |
| Missing   | 6       | 5        |        |
Discussion

Over the last decade, with the extensive development of genomic transcription study, accumulative studies indicated that lncRNA have risen to prominence with important roles in a broad range of biological processes. Recent studies have reported that several lncRNAs, for example, MALATA, CASC15, LOC440896, LINC00632, IGF2-AS have been implicated in characteristics and prognosis of neuroblastoma [5,6,11]. Therefore, lncRNA have the potential to serve as novel biomarkers for neuroblastoma diagnosis or prognosis. However, the biological functions of most lncRNAs have yet to be explored. High-risk neuroblastoma patients often have unfavorable outcomes, it is one of the biggest obstacles to improve overall survival of neuroblastoma. Hence, it is urgent to investigate the novel genes and illustrate the molecular mechanisms of neuroblastoma. The main purpose of this study was to investigate the differential expression of LRRC75A-AS1 in neuroblastoma, and to find new prognostic and diagnostic markers for neuroblastoma. The results of this study show that LRRC75A-AS1 is up-regulated in neuroblastoma.

Small nucleolar RNA host gene 29 (SNHG29) was also known as LRRC75A-AS1, TSAP19, C17orf45, NCRNA00188, FAM211A-AS1, C17orf76-AS1, it’s a long noncoding RNA that leucine rich repeat containing 75 A-antisense RNA1 is located on 17p11.2 [12]. Emerging reports has revealed that LRRC75A-AS1 was involved in several biological processes through modulation of signaling pathway. Jeong et al. [13] reported that LRRC75A-AS1 can regulate the vascular calcification negatively, and might act as a possible target in the treatment of vascular calcification. Wang et al. [14] found that LRRC75A-AS1 can regulate the expression of tight junction (TJ) proteins through LRRC75A, affecting the inflammatory responses of bovine mammary epithelial cells. Leavey K et al. [15] show that LRRC75A is abnormally expressed in the process of normal villous maturation.

In cancers, LRRC75A-AS1 has been served as a crucial regulator in a variety types of cancers including osteosarcoma [12,16], colorectal carcinoma [17], breast cancer [18], gastric cancer [19], glioblastoma [20] and acute myeloid leukemia [21]. Joeri Both et al. [12,15] claimed that LRRC75A serves as malignant facilitator in osteosarcoma. Jianxiong Chen et al. [16] reported that LRRC75A-AS1 inhibits cell proliferation and migration in colorectal carcinoma, and it might serve as an anti-oncogene for colorectal carcinoma, and it might serve as an anti-oncogene for colorectal carcinoma (CRC) tumorigenesis and advancement. Lizhang Han et al. [20] found that SNHG2(LRRC75A-AS1) can regulates miR-223-3p/CTNND1 axis to promote glioblastoma progression via Wnt/β-catenin signaling pathway. FANGCE WANG et al. [21] proved that LRRC75A-AS1 can significantly predict prognosis of acute myeloid leukemia. These papers showed that LRRC75A-AS1 may become a novel molecular marker for diagnosis and treatment of cancer. However, little information of the prognostic value and the role of LRRC75A-AS1 in neuroblastoma has been reported.

In the present study, we compare the expression profile of lncRNA between 4 III phase and 4 IV phase tumor tissues of neuroblastoma by using the RNA-sequencing. We found that LRRC75A-AS1 was the overexpressed lncRNA in IV stage patients with the fold change of 3.19. To further confirm the relationship of LRRC75A-AS1 in neuroblastoma, the RT-qPCR analysis was used.
to analyze the clinical tissue from neuroblastoma patients. These experimental results showed that LRRC75A-AS1 was obviously high in advanced stage neuroblastoma, and high expression level of LRRC75A-AS1 was associated with advanced stage disease, high risk group, N-MYC Amplified, unfavorable histology, and the level of LDH, D-Dimer and NSE, which are strong predictors for prognosis of neuroblastoma. Furthermore, we used public neuroblastoma dataset in R2 validated that overexpression of LRRC75A-AS1 was correlated with unfavorable prognosis in neuroblastoma. But, the expression of LRRC75A-AS1 was not correlated with ki-67 level, Vanilla mandelic acid (VMA) level and tumor size, the inconsistency may be caused by the small sample size of this study which did not permit attainment of statistical significance.

The limitation of current experiments is the small sample size resulting in limited statistical power, hence, it is still necessary to expand the clinical sample size and patients should also be long-term followed up to validate the prognosis in the public neuroblastoma dataset in our cohort. This study merely proved the relationship between LRRC75A-AS1 and tumor in clinic, so more relevant basic experiments should be practiced on animals and cells. Further studies are needed to illuminate the underlying molecular mechanisms that LRRC75A-AS1 might promote the tumorigenesis and progression of NB, as well as screening for potential therapeutic target for neuroblastoma.

**Conclusion**

In conclusion, our study demonstrated that LRRC75A-AS1 was up-regulated in IV phase tumor. Further experiments revealed that overexpression of LRRC75A-AS1 in tumor tissues was associated with aggressive disease including INSS III, IV stage, high risk group, N-MYC amplified, uFH classification, high level of LDH, D-Dimer and NSE, and unfavorable overall survival. Thus LRRC75A-AS1 may function as a potential prognostic biomarker in neuroblastoma, and we conjecture a novel prognostic model including LRRC75A-AS1 and tumor in clinic, so more relevant basic experiments should be practiced on animals and cells. Further studies are needed to illuminate the underlying molecular mechanisms that LRRC75A-AS1 might promote the tumorigenesis and progression of NB, as well as screening for potential therapeutic target for neuroblastoma.

**Statements**

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**Statement of Ethics**

This research complies with the guidelines for human studies. This study approved by the ethics committee of the Children’s Hospital of Chongqing Medical University and subjects (or their parents or guardians) have given their written informed consent.

**Disclosure Statement**

The authors have no conflicts of interest to declare.

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**Author Contributions**

WZ and WS designed the experiments, analyzed the data and revised the manuscript. WZ wrote the manuscript. WZ performed most of the experiments. LC, YC, PL and SJ collected tumor tissues and performed the experiments. All of the authors discussed the results and reviewed the manuscript.

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