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Research paper

Expression and function of lncRNA MALAT-1 in the embryonic development of zebrafish

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ABSTRACT

Long non-coding RNAs (lncRNAs) are RNA molecules with a transcript length of > 200 nt which do not encode proteins, however, they have important functions in the form of RNA at transcriptional level, post-transcriptional level, and epigenetics. MALAT-1 is the first discovered lncRNA in non-small cell lung cancer and its orthologs have been found in zebrafish in the recent years. The present study aimed to determine the expression and possible function of MALAT-1 in the embryonic development of zebrafish. The results of qPCR or in situ hybridization showed that MALAT-1 was dynamically expressed in zebrafish embryonic development and it was widely expressed in brain, eye, heart, and muscle of adult fish. By morpholino knockdown of MALAT-1, zebrafish embryos were found to have smaller body curvature, smaller eyes, enlarged pericardium, and reduced pigmentation. In particular, after morpholino knockdown of MALAT-1, the morphologically defective otic capsule was found to be smaller than the control fish. These results suggest that MALAT-1 may play a key role in the embryonic nervous system development of zebrafish. In addition, we also determined the expressions of seven development-related genes (*Scyl1*, *egr1*, *oc90*, *vsx1*, *nkx2.5*, *vmhc*, and *gata4*) after MALAT-1 knockdown and found that four genes *egr1*, *nkx2.5*, *gata4*, and *vmhc* were up-regulated, suggesting a potential regulatory role of MALAT-1 on these development-related genes.

1. Introduction

In the recent years, with the development of genomics, bioinformatics, and the massive application of high-throughput sequencing technology, it has been found that only < 2% of the transcripts in human genome have protein coding function, most of which are non-coding RNA (ncRNA) such as tRNA, rRNA, miRNA, and lncRNA (Adams et al., 2017). Now ncRNA have attracted more and more attention due to their functions in the gene expression regulation. Long non-coding RNAs (lncRNAs) are the ncRNA molecules with a transcript length of > 200 nt and transcribed by RNA polymerase II (Ponting and Belgard, 2010). According to the different ways of transcription and localization of lncRNAs, they are roughly divided into the following five types: intergenic lncRNA, bidirectional lncRNA, intron lncRNA, sense lncRNA, and antisense lncRNA (Ponting et al., 2009).

Accumulated studies have shown that lncRNAs have been implicated in numerous biological processes in organisms including cell proliferation, differentiation, metabolism, and apoptosis (Hung et al., 2011), embryonic development (Chambers et al., 2003), cancer (Luo et al., 2013), and other diseases (Huang et al., 2013). They can regulate target genes at the transcriptional, post-transcriptional, and epigenetic levels, respectively (Gutschner and Diederichs, 2012). The regulation of all physiological or pathological processes is closely related, so the elucidation of the function and regulation mechanism of lncRNA is of great significance to modern life sciences.

Embryonic development is a complex process which is regulated by many transcriptional regulatory factors. With the development of high-throughput deep sequencing technology, it has been found that lncRNA plays a very important role in the regulation of animal embryonic development. Dinger et al. (2008) found two developmentally related lncRNAs, Evx1as and HOXB5/6AS, in mouse ES cells. Their CHIP experiments showed that the two LncRNAs were involved in histone

Abbreviations: AS, alternative splicing; B-MYB, Myb-related protein B; Egr1, early growth response 1; hpf, hour post-fertilization; lncRNA, long non-coding RNA; MALAT-1, metastasis-associated lung adenocarcinoma transcript 1; mascRNA, MALAT-1-associated small cytoplasmic RNA; MO, Morpholino; ncRNA, non-coding RNA; NEAT2, nuclear-enriched autosomal transcript 2; NSCLC, non-small cell lung cancer; Oc90, Otoconin-90; qPCR, quantitative realtime PCR; SMAD, Drosophila mothers against decapentaplegic protein; SR, serine/arginine; Vsx1, visual system homeobox-1

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methyltransferase and trimethylated H3K4 histones, indicating that lncRNA has a regulatory role in the development and differentiation of ES cells. H19 is the earliest discovered lncRNA and highly expressed in the embryonic stage of mice. With the birth of mice, the expression level of H19 was significantly reduced, but the gene maintained a strong expression signal in mouse skeletal muscle before and after birth. Therefore it may play a crucial role in the development of skeletal muscles. Further studies have demonstrated that LncRNA-H19 encodes miR-675-3p and miR-675-5p, which directly interacts with the transcription factor SMAD (Drosophila mothers against decapentaplegic protein), thereby regulating bone development (Steck et al., 2012). Interestingly, a recent study in zebrafish identified 29 evolutionarily conserved lncRNAs, of which two lncRNAs exhibited a significant regulatory function during zebrafish development as demonstrated by morpholino intervention (Ulitsky et al., 2011).

The metastasis-associated lung adenocarcinoma transcript 1 (MALAT-1), also known as nuclear-enriched autosomal transcript 2 (NEAT2), is an important member of the lncRNA family. It was originally discovered in the study of non-small cell lung cancer (NSCLC) in 2003 (Ji et al., 2003). MALAT-1 is specifically localized in the nuclear speckle region of the nuclear nucleosome and is transcribed in this region, but its enrichment in this nuclear plaque presupposes that RNA polymerase II-dependent transcription is activated (Clemson et al., 2009). Phylogenetic analysis indicates that MALAT-1 is highly conserved among mammals (Zhang et al., 2012), however, compared with MALAT-1 in zebrafish, it has only similarity at the 3′ end. Previous studies have shown that nuclear-enriched MALAT-1 interacts with serine/arginine (SR) protein, affecting the distribution of splicing factors. Silencing MALAT-1 or over-expressing SR protein can alter the alternative splicing of endogenous pre-mRNA to varying degrees (Tripathi et al., 2010). The study also found that MALAT-1 regulated the phosphorylation and dephosphorylation of SR proteins at the cellular level, thus controlling their activity. In addition, MALAT-1 also can bind to demethylated Polycomb 2 to promote the relocation and expression of growth genes (Bernstein et al., 2006). Moreover, MALAT-1 has shown to regulate the expressions of cell cycle related genes. B-MYB (Myb-related protein B) is an oncogenic transcription factor involved in the progression of G2/M phase, in the cells silenced by MALAT-1, the splicing factor binding site on the precursor mRNA of B-MYB changes and abnormal splicing occurs (Tripathi et al., 2013). Additionally, MALAT-1 is highly expressed in various tumors such as colorectal cancer (Yang et al., 2015), liver cancer (Wang et al., 2014), and breast cancer (Zhao et al., 2014), and it can promote tumor cells proliferation, metastasis, and invasion.

Currently, there have been only few reports on the function of MALAT-1 in the embryonic development of zebrafish. In the present study, we first determined the expression of MALAT-1 in the embryonic development of zebrafish by quantitative realtime PCR (qPCR) and in situ hybridization. To evaluate the possible function of MALAT-1, specific morpholino was designed to knock-down MALAT-1 and then to determine its effect on the embryonic development. This study provides the clue for further exploration of the role of MALAT-1 in embryonic development.

2. Materials and methods

2.1. Experimental animals

Zebrafish used in the experiment was provided by the Animal Center of Qixiu Campus of Nantong University, China. The rearing water was tap water filtered by activated carbon. The oxygen saturation is > 80% and the temperature is 28 ± 1 °C. The domesticated photoperiod is 14:10 h of day: night and the illumination is 1000 lx. Spawning was stimulated by turning on the light and the healthy fertilized eggs/embryos were collected under stereomicroscope at 2, 6, 10, 12, 24, 36, 48, and 72 h after fertilization, respectively. Adult fish were dissected under a stereomicroscope.

Table 1
Specific primers used for qPCR in the present study.

Primers	Sequences
MALAT-1-qPCR-F	5′-AAACCCATCCACTCGCTCTG-3′
MALAT-1 qPCR-R	5′-CTTATCTCCAGGTAGCGGCG-3′
MALAT-1-Probe-F	5′-GATCATTGGGTGGGTGTAGG-3′
MALAT-1-Probe-R	5′-TCACAGACAGCCTTGCATTC-3′
MALAT-1-Test primer-F	5′-AGTTGAACCCCGTCCTGAGA-3′
MALAT-1-Test primer-R	5′-AAGGCGTGTGAATATGCTGC-3′
nkx2.5-F	5′-CTTCTCTCAGGCGCAGGT-3′
nkx2.5-R	5′-GGATGCTGGACATGCTCGACGGA-3′
gata4-F	5′-TGCAGAAGGAGAGCCAGTCT-3′
gata4-R	5′-ATGCTGGAAACGCAGATACC-3′
vmhc-F	5′-GATGGCAGAGTTTGGAGCAG-3′
vmhc-R	5′-AACTTTGGAGGGTTCTGGGG-3′
egr-1-F	5′-GACACCTGACGACCCACATT-3′
egr-1-R	5′-AAGACTGGAGACGGGAAAGAG-3′
oc90-F	5′-CCAGCCAGCGCAGGTATGTGTA-3′
oc90-R	5′-GCGGCTGAGGAAACTCGAAGATC-3′
vsx1-F	5′-AGCCAGCAGGAATGCACAA-3′
vsx1-F	5′-GAATCGTCCGCTCCATTAG-3′
Rpl13α-F (Tang et al., 2010)	5′-TCTGGAGGACTGTAAGAGGTATGC-3
Rpl13α-R	5′-AGACGCACAATCTTGAGAGCAG-3
Scyl1-F	5′-ACTGAAAGTGGGGGTTTGGA-3′
Scyl1-R	5′-CTGAGGGTTTTTGTGCGCTT-3′

F, forward, R, reverse.

2.2. RNA isolation, reverse-transcription, and qPCR

When detection of MALAT-1 expression at different stages of zebrafish embryos, 90 healthy embryos were taken at each corresponding time points as described above and then randomly divided into 3 groups in which 30 embryos were included per sample for RNA isolation. For adult, totally 9 fish were three groups (3 fish in each group), and then all fish were dissected and the organs of brain, eyes, heart, liver, kidneys, spleen, and muscle were taken for RNA isolation. Three organs from one group were put together for enough amount of RNA. Total RNA was extracted by using Trizol reagent (Vazyme) according to the manufacturer's protocol. cDNA was produced with Transcriptor High Fidelity cDNA Synthesis Kit (Roch) using 1 μg of total RNA per reaction. qPCR reaction was performed in triplicate with SYBR green mix in a Light Cycler 480 (Roche). *Rpl13α* gene was used as endogenous reference control. Primers used in qPCR are listed in Table 1.

2.3. In situ hybridization

Whole embryonic in situ hybridization was used to detect the spatial expression of MALAT-1. Totally 20 healthy embryos were taken at the corresponding eight time points as described above and whole embryo in situ hybridization was performed after fixation according the method of Cheng et al. (2014) using digoxigenin as a hapten and NBT/BCIP as a color substrate. Each test was performed in triplicate.

2.4. Morpholino injection

Antisense morpholino oligonucleotide (MO) designed by Gene Tools was complementary to the region of the splicing site (MALAT-1-MO: CCACCAGGGTCTTTTGCTTTTTTTC). Totally 300 embryos of the fish were used for microinjection of MO knockdown MALAT-1, while 300 embryos with the same developmental stage were injected the same volume of “standard morpholino oligomers”, not targeting any gene in zebrafish embryos, as controls. MOs were injected into the yolk of one cell stage embryos and they were then cultured at 28 °C in 1× E3 embryo medium with 0.05% methylene. Embryos were injected with MOs at concentrations of 250, 300, and 350 μM according to the result of the preliminary experiment and in the end 300 μM was adopt as the