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Original article

The long non-coding RNA MALAT1 interacted with miR-218 modulates choriocarcinoma growth by targeting Fbxw8

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ABSTRACT

Among the first found cancer-related long non-coding RNAs (lncRNAs), MALAT1 is one that involves in the development and progression of some tumors. MALAT1 can be aberrantly expressed in hepatocellular carcinoma, cervical, breast, ovarian cancers, as well as colorectal cancer. The paper aims to make certain the function of MALAT1 in human choriocarcinoma cell lines by investigating the detailed effects and molecular mechanisms. Being specifically upregulated in choriocarcinoma cell lines, the under-researched lncRNA-MALAT1 promoted choriocarcinoma cell growth by targeting miR-218. After MALAT1 knockdown, proliferation of human choriocarcinoma cell *in vitro* was dramatically hindered, and the tumor size *in vivo* was reduced. What is more, miR-218-mediated Fbxw8 regulation was required for MALAT1-induced choriocarcinoma cell proliferation. Taken together, MALAT1 might promote choriocarcinoma tumor growth through miR-218-mediated Fbxw8 regulation. According to our data, MALAT1 might be an oncogenic lncRNA that promoted choriocarcinoma proliferation and could be therapeutically targeted in human choriocarcinoma.

1. Introduction

Being commonly associated with gestation, it is common to identify choriocarcinoma in females at the origin of the chorionic epithelium of the placenta. As the tumor rapidly grows, widely metastasizes, the surrounding tissues are extensively invaded. Gonadal choriocarcinomas have shown great sensitiveness in respond to a great variety of anticancer agents [1]. Recently chemotherapy and radiation therapy are usually relied on to deal with choriocarcinoma clinically [2]. For the vast majority of patients, it is feasible to apply chemotherapy to choriocarcinoma treatment for a prolonged period of time and in some cases the clinical cure occurs. However, due to the aqucision of chemoresistance to chemo-agents as well as side effects after chemotherapy which makes it impossible to complete chemotherapy treatments, it is not uncommon to undergo chemotherapy failures. In such case, it is impossible to estimate the condition and prognosis of patients clinically. Therefore, it has been urgent to find out new methods for the diagnosis, treatment as well as prognosis of choriocarcinoma.

Being especially essential in both initiation and development of tumors, microRNAs (miRNAs) serve as either oncogenes or tumor suppressors, depending on circumstances [3,4]. MiRNAs are reportedly associated with choriocarcinoma tumorigenesis [5,6]. Among numerous miRNAs, miR-218 participates in the development as well as progression of many cancers, such as breast cancer [7], osteosarcoma [8] and colorectal cancer [9]. Yuan et al. reported that miR-218 is greatly downregulated in NSCLC tissues and cell lines; miR-218 overexpression significantly represses the tumor growth and metastasis through the regulation of IGF1R [10]. Based on these studies, it can be conferred that miR-218 may act as a tumor suppressor in these cancers; while, in fact, how it works and the possible mechanisms in choriocarcinoma have remained unclear.

The genome sequence data in human beings indicates that over 90% of the DNA sequences are transcribed actively, but merely 2% of them encodes protein, therefore, most of transcripts are referred to as non-coding RNAs (ncRNAs) [11,12]. There have been extensive studies with regard to small non-coding RNAs including microRNAs whose roles in regulation of gene and function of cell have been illustrated in various cancers [12]. According to recent studies, lncRNAs have been essential in not only usual development but also diseases including cancers [13]. Recently, LncRNAs have be considered by several studies to serve as, in different circumstances, either oncogenes, or tumor suppressor genes or both [14].

LncRNAs can exert their functions through diverse mechanisms, among which the interaction between lncRNAs and microRNAs has been regarded as the most important one [15,16]. The metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), also known as MALAT-1 or nuclear-enriched abundant transcript 2 (NEAT2), was originally identified via subtractive hybridization as a prognostic

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parameter for patient survival of stage I lung adenocarcinoma or squamous cell carcinoma patients [17–19]. MALAT1 was one of the first identified ncRNAs associated with lung cancer; it was first discovered as a prognostic marker for lung cancer metastasis but also has been linked to several other human tumor entities in recent decades [20]. For example, MALAT1 not only reflected the prognosis of colorectal cancer to be poor but also promoted tumor growth by means of recruiting and binding to eIF4A3 [21]. In addition, MALAT1 overexpression can also promote the invasion, angiogenesis, as well as stemness of glioblastoma cells [22]. However, the detailed role of MALAT1 in choriocarcinoma still remains unclear.

In general, the present study demonstrated an interaction of MALAT1 and miR-218 which modulates choriocarcinoma cell proliferation by means of directly targeting F-box/WD repeat-containing protein 8 (Fbxw8). Based on the findings, a new proposal of the role of MALAT1 and miR-218 in choriocarcinoma growth as well as the mechanism has been provided.

2. Materials as well as methods

2.1. Cell lines and cell transfection

We purchased such human choriocarcinoma cell lines as JEG-3, JAR and BeWo cells and a normal cell line, human trophoblast cells (HT cells), from Manassas, VA, USA. They were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) with a supplementation of 10% fetal bovine serum (Gibco, CA, USA) at 37 °C in a humidified atmosphere with 5% CO_2. The miR-218 expression was realized by transfection of miR-218 mimics or miR-218 inhibitor (Genepharma, Shanghai, China) with the use of Lipofectamine 2000 (Invitrogen). Si-MALAT1 was used to achieve knockdown of MALAT1 (GeneCopoecia, Guangzhou, China). Being plated in 6-well or 96-well plates, cells were transfected and incubated for 48 h so as to be used for further assays or RNA/protein extraction.

2.2. RNA extraction and SYBR green quantitative PCR analysis

We extracted total RNA from cells with the use of Trizol reagent (Invitrogen, CA, USA) and applied a Hairpin-it TM miRNAs qPCR kit (Genepharma, Shanghai, China) to detect mature miR-218 expressions in cells. Expression of RNU6 B was used as an endogenous control. SYBR green qPCR assay (Takara, Dalian, China) was applied to measure MALAT1 expression and $2^{-\Delta\Delta CT}$ method was adopted for the process of the data.

2.3. MTT assay

To make an evaluation of cell viability, a modified MTT assay was applied. With 2×10^3 transfected cells/well seeded into 96-well culture plates containing the indicated medium (RPMI-1640 medium with a supplementation of 10% fetal bovine serum) we assessed the viability of JEG-3 and JAR cells at five time points (on day 1, 2, 3, 4 and 5). In brief, to achieve quantification of mitochondrial dehydrogenase activity, the enzymatic conversion of MTT [3-(4, 5-dimethyldiazol-2-yl)-2, 5- diphenyltetrazolium bromide; Sigma-Aldrich, MO, USA] to a colored formazan product was applied. Being added to the cells, MTT (10 μl, 10 mg/ml) was incubated for 4 h, and the reaction was terminated when we removed the supernatant and added 100 μl DMSO to dissolve the formazan product. After 0.5 h, the optical density (OD) of each well stood at 490 nm with the use of a plate reader (ELx808 Bio-Tek Instruments, City, ST, USA).

2.4. Flow cytometry assay for cell cycle analysis

In accordance with the manufacturer's protocol, after 48 h of transfection with Click-iT EdU Alexa Fluor 647 Flow Cytometry Assay Kit (Thermo Fisher Scientific Inc.), JAR and JEG-3 were processed. Then it was time to stain cells with propidium iodide and perform and analyze cell cycle analysis on a Navios flow cytometer (BeckmanCoulter) using Kaluza software (v1.2, Beckman Coulter).

2.5. Cell scratch test

The cells were respectively digested, and the cell concentration was adjusted to 5×10^5 cells/mL. Cell suspensions (100 μl) were plated in 96-well plates coated with Metrigel, routinely cultured until the cell monolayer emerged. Then the cell scratch test was performed. The cells were cultured in RPMI-1640 supplemented with 10 g/L bovine serum albumin (BSA) and 1% FCS, and the scratch area was measured under the microscope. 24 h later, the cells continued to culture for another 24 h in RPMI-1640 supplemented with 10% FCS, and then the relative distance of cell migration to injury area was also measured under the microscope.

2.6. Western blot analysis

The expressions of Fbxw8, Cyclin A and p27 in choriocarcinoma cells were detected by performing Immunoblotting. We lysed cultured or transfected cells in RIPA buffer with 1% PMSF, and loaded protein onto a SDS-PAGE minigel and the cells were transferred onto PVDF membrane. With 1:1000 diluted rabbit polyclonal Fbxw8, Cyclin A and p27 antibody (Abcam, MA, USA) at 4 °C overnight, we probed the blots which were subsequently incubated with HRP-conjugated secondary antibody (1:5000). ECL Substrates was used to visualize signals (Millipore, MA, USA). We use β-actin as an endogenous protein for normalization. The results from Western blot analysis were quantified using Image J software (NIH).

2.7. Luciferase reporter assay

MALAT1 fragment containing putative miR-218 binding site predicted by miRanda software was amplified by PCR using the primers shown in Supplemental material. The amplified MALAT1 fragment was then cloned to the downstream of the Renilla psiCHECK2 vector (Promega, Madison, WI, USA, structure shown in Fig.S1 in Supplemental materials), named wt-MALAT1. To generate mutant MALAT1 reporter, the MALAT1 fragment was mutated to remove all complementarity to nucleotides 2–11 of miR-218, named mut-MALAT1. All sequences of primers were shown in Supplemental material. JEG-3 cells were seeded into a 24-well plate. After cultured overnight, JEG-3 cells were co-transfected with the indicated vectors and miR-218 mimics or miR-218 inhibitor, respectively. Luciferase assays were performed 48 h after transfection using the Dual Luciferase Reporter Assay System (Promega). Renilla luciferase activity was normalized to Firefly luciferase activity for each transfected well.

2.8. Xenograft tumor model

BALB/c-nu mice (4 ~ 5 weeks of age and weighing 15 to 18 g) were purchased from the Guangdong Experimental Animal Center, and their care was in accord with institutional guidelines. The mice were randomly divided into two groups (6 mice in each group). One group of mice was inoculated subcutaneously with 5×10^6 JEG-3 cells expressing the vector control (si-NC) and the other with 5×10^6 JEG-3 cells expressing si-MALAT1. After 4 weeks of observation, the mice were sacrificed and tumors were obtained [23]. The tumor volumes and weights were determined. The tumor volume was calculated according to the formula: tumor volume (mm^3) = length (mm) × width (mm) × height (mm) [24], and the tumors were paraffin-embedded.