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RESEARCH ARTICLE

MALAT1-miR-124-RBG2 axis is involved in growth and invasion of HR-HPV-positive cervical cancer cells

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Abstract Metastasis-associated lung adenocarcinoma transcript 1 (MALAT 1) is a large, infrequently spliced noncoding RNA aberrantly expressed in cervical cancer. But the molecular mechanisms of its oncogenic role are still not quite clear. The present study explored whether there is a competing endogenous RNAs (ceRNAs) mechanism involved in the oncogenic effect of MALAT1. MALAT1 expression was firstly verified in high-risk human papillomavirus (HR-HPV)-positive tumor tissues and cell lines. Its regulation over miR-124 and the downstream target of miR-124 in regulation of growth, invasion, and apoptosis of the cancer cells are also studied. Findings of this study confirmed higher MALAT1 expression in HR-HPV (+) cervical cancer. Knockdown of endogenous MALAT1 significantly reduced cell growth rate and invasion and increased cell apoptosis of Hela and siHa cells. Besides, knockdown of MALAT1 increased the expression of miRNA-124, while ectopic expression of miR-124 decreased MALAT1 expression. In addition, we also verified a direct interaction between miR-124 and 3′UTR of GRB2. MALAT1 can indirectly modulate GRB2 expression via competing miR-124. Knockdown of GRB2 reduced cell invasion and increased cell apoptosis. In conclusion, MALAT1 can promote HR-HPV (+) cancer cell growth and invasion at least partially through the MALAT1-miR-124-RBG2 axis. This finding might provide some useful evidence about the lncRNA interaction regulatory network in tumorigenesis cervical cancer.

Keywords MALAT1 · miR-124 · GRB2 · HR-HPV · Cervical cancer

Introduction

Cervical cancer is the third most common malignancy in women [1]. Persistent infection of high-risk human papillomavirus (HR-HPV), typically HPV-16 and HPV-18, is the main risk factor of cervical cancer development [2]. In fact, infection of HPV-16 and HPV-18 account for about 70 % of cervical cancer cases [3]. HR-HPV infection leads to a series of dysregulated molecular processes which contribute to cervical carcinogenesis [4, 5]. But the exact mechanism of the changes was not fully understood.

Long non-coding RNAs (lncRNAs) are non-coding transcripts with over 200 nucleotides in length. There are emerging evidence showing that they are important molecules in both normal development and tumorigenesis [6–8]. Metastasis-associated lung adenocarcinoma transcript 1 (MALAT 1) is a large, infrequently spliced non-coding RNA aberrantly expressed in cervical cancer [9, 10]. Inhibition of MALAT1 in CaSki, a HPV-16-positive human cervical cancer cell line, can suppress cell proliferation and invasion [10]. However, the oncogenic role of MALAT1 is still not quite clear.

MiR-124 is generally considered as a tumor suppressor in several types of cancer, such as hepatocellular carcinoma [11], gastric cancer [12], and cervical cancer [13]. Low miR-124 expression is associated with higher vasculogenic mimicry, migration, and invasion of the HR-HPV positive (+) cervical cancer cells [13]. SNP and methylation are two mechanisms of lowered miR-124 expression in cervical cancer [14, 15]. But whether other mechanism is involved in reduced miR-

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124 expression and what are the downstream targets of miR-124 should be further studied.

In the current study, we reported a novel regulation of MALAT1 over the expression of miR-124 and verified a new target of miR-124, GRB2, in regulating growth and invasion of HR-HPV (+) cervical cancer cells.

Methods

Human tissue samples

This study was approved by the ethics committee of Cangzhou Central Hospital. Cervical tissue samples were obtained from 22 patients histologically diagnosed as IA with lyphovascular space invasion (IVSI) or IA2 cervical cancer, as described in our previous study [16]. Twenty-two cases of HR-HPV-negative (−) normal cervical squamous epithelium were obtained from patients who had HR-HPV test and cervical Thinprep cytological test at Cangzhou Central Hospital. Informed consent was obtained from each participant.

Cell culture

Human cervical cancer cell lines, HeLa (HPV18-positive), CaSki (HPV16-positive), and SiHa (HPV16-positive) were grown in RPMI-1640 medium (Gibco-BRL, USA) supplemented with 10 % fetal bovine serum (HyClone, USA). All cells were cultured in a humidified atmosphere containing 5 % CO_2 at 37 °C.

Cell transfection

Hela and siHa cells were transfected with two MALAT1 siRNAs individually [100 nM, si-MALAT1 (no. 1 and no. 2); QIAGEN] or negative control siRNA [si-NC; QIAGEN] according to the manufacturer's instructions. The effect of MALAT1 knockdown was assessed using qRT-PCR 48 h after transfection. The siRNA with higher inhibitive effect was used for following studies. To overexpress miR-124, Hela and siHa cells were transfected with miR-124 mimics and the negative control (75 nM, Ribo Life Science) using Lipofectamine 2000 (Invitrogen). The effect of miR-124 overexpression was assessed using qRT-PCR 24 h after transfection. Cell viability at 24, 48, and 72 h after transfection was measured using Cell Counting Kit-8 (CCK-8) (Dojindo, Japan) according to manufacturer's instruction. To knockdown of GRB2 expression, Hela and siHa cells were transfected with 200 nM si-GRB2 (Ribo Life Science) using Lipofectamine 2000 (Invitrogen).

Transwell analysis of cell invasion

Cell invasion assay was performed using the Transwell insert chamber coated with Matrigel (BD Biosciences). Briefly, 1×10^5 cells were suspended in 200 μL serum-free RPMI-1640 medium and then plated into the upper chamber. To create chemoattractant environment in the lower chamber, it was filled with RPMI-1640 supplemented with 20 % FBS. After 24 h incubation in a cell incubator, cells on the top surface of the insert were removed. The cells on the bottom surface were fixed with 4 % polyoxymethylene, and the number of invading cells was counted after staining with 0.1 % crystal violet. Each experiment was performed in triplicate.

Flow cytometry analysis of apoptotic cells

Forty-eight hours after transfection, cells were harvested and fixed in 70 % ice-cold ethanol at 4 °C for 24 h. The ratio of cells with active caspase-3 was measured using Fluorescein Active Caspase 3 Staining Kit (Abcam, ab65613) in a flow cytometer (FACSCalibur, BD Biosciences).

qRT-PCR analysis of MALAT1, GRB2, and miR-124 expression

Total RNA in the cell and tissue samples were extracted using the TRIzol reagent (Invitrogen, USA) according to manufacturer's instructions. cDNA was reversely transcribed using a First Strand Synthesis kit (Invitrogen). qRT-PCR analysis of MALAT1 and GRB2 was performed using the gene-specific primers (MALAT1: forward, 5′-AAAGCAAGGTCTCCCCACAAG-3′, reverse, 5′-GGTCTGTGCTAGATCAAAAGGCA-3′; GRB2: forward, 5′-TTGTGTGTCCCAGTGTGCAA-3′, reverse, 5′-AGCTCAGCTCATCGTCAGCA-3′) and Power SYBR Green PCR Master Mix. GAPDH served as the endogenous control. qRT-PCR analysis of miR-124 was performed using TaqMan MicroRNA Assay Kit (Applied Biosystems) with U6 snRNA served as the endogenous control. All qRT-PCR analysis was performed in an ABI Prism 7500 (Applied Biosystems). The expression change was calculated using $2^{-\Delta\Delta CT}$ method.

Dual luciferase assay

Wild-type (WT) or mutant (MUT) sequence of GRB2 3′UTR containing the predicted miR-124 binding site was chemically synthesized and inserted between the PmeI and XbaI sites in the downstream of the firefly luciferase gene in pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega). The recombinant vectors are named as pmirGLO-GRB2-WT and pmirGLO-GRB2-MUT, respectively. Hela and siHa cells were co-transfected with 200 ng reporter plasmids and 50 nM miR-124 mimics. Twenty-four

hours after transfection, both firefly and renilla luciferase activities were measured by the Dual-Luciferase Reporter Assay System (Promega) using a Promega GloMax 20/20 luminometer. The firefly luciferase activity was normalized to the renilla luciferase activity.

Western blot analysis of GRB2 expression

Total protein from tissues and cells were extracted by using RIPA buffer (50 mM TrisHCl, 150 mM NaCl, 2 mM EDTA, 1 % NP-40, and 0.1 % SDS). Total protein concentration was measured by using BCA protein assay (Pierce, Thermo Scientific) and then separated on 10 % SDS PAGE gel and transferred onto nitrocellulose membranes for a conventional western blot analysis. GRB2 protein was detected with Anti-GRB2 (1:1000, ab2234, Abcam). GAPDH served as loading control and was detected by using ant-GAPDH (1: 2000, ab125247, Abcam). Membranes were washed and incubated with corresponding HRP-labeled secondary antibodies. Protein signals were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and intensity of each band was quantified by ImageQuant 5.2 (GE Healthcare, Piscataway, NJ).

Statistical analysis

Data were presented as mean±SD with at least three repeats. Group comparison was performed by unpaired *t* test. MALAT1 and miR-124 expression in the three cervical cancer cell lines was compared with the average level of the whole control group. *P* value <0.05 was considered as significant difference; *, **, and *** donates significance at 0.05, 0.01, and 0.001 level respectively, while NS indicates not significant.

Results

MALAT1 expression is significantly upregulated in HR-HPV (+) cervical cancer

MALAT1 is an lncRNA generally upregulated in cervical cancer. In the current study, we also observed that MALAT1 is significantly upregulated in HR-HPV (+) cervical cancer tissues than in normal tissues (Fig. 1a). Besides, MALAT1 expression in HPV16-positive CaSki and SiHa cells and in HPV18-positive HeLa cells was quantified. Compared with normal tissue samples, MALAT1 expression was significantly higher in these three HR-HPV (+) cervical cancer cell lines (Fig. 1b). These results suggest that MALAT1 expression is significantly upregulated in HR-HPV (+) cervical cancer.

MALAT1 involves in regulation of cell growth and invasion of HR-HPV (+) cervical cancer cells

To explore the biological functions of MALAT1 in HR-HPV (+) cervical cancer cells, Hela and siHa cells were firstly transfected with MALAT1 siRNA (Fig. 2a, b). Knockdown of endogenous MALAT1 significantly reduce cell viability of both Hela and siHa cells (Fig. 2c, d). In addition, Hela and siHa cells with lowered MALAT1 expression also had significantly reduced cell invasion capability (Fig. 2e, f) and increased apoptosis (Fig. 2g, h). These results suggest that MALAT1 involves in regulation of cell growth and invasion of HR-HPV (+) cervical cancer cells.

MALAT1 regulates cell growth and invasion through interacting with miR-124

Through our preliminary screening, we found that several miRNAs were significantly downregulated in HR-HPV (+) cancer (data not given). Based on quantification and regression analysis of MALAT1 and miR-124 expression in the 22 cancer cases, we observed that miR-124 expression was negatively correlated to MALAT1 expression in cervical cancer tissues (Fig. 3a). Our bioinformatics analysis based on StarBase 2.0 [17] found that MALAT1 has two putative binding sites with miR-124 (Fig. 3b). The low miR-124 expression is also confirmed in the Hela, siHa, and CaSki cells (Fig. 3c). Knockdown of MALAT1 significantly reduced the mature miR-124 level in both Hela and siHa cells (Fig. 3d). Interestingly, enforced miR-124 expression in these two cells also reduced MALAT1 level (Fig. 3f). Therefore, there is a reciprocal interaction between MALAT1 and miR-124. Overexpression of miR-124, similar as knockdown of MALAT1, could decrease cell viability (Fig. 3g, h) and cell invasion capability (Fig. 3i) and increase the ratio of apoptotic cells (Fig. 3j) of both Hela and siHa cells. Combined MALAT1 knockdown and miR-124 overexpression have some level of synergetic effect. These results suggest that MALAT1 can regulate cell growth and invasion through interacting with miR-124.

MiR-124 directly targets GRB2 and regulates its expression, thereby affecting cell invasion and apoptosis

MiR-124 is generally considered as a tumor suppressor in cervical cancer. But its downstream regulation is not fully understood. Since the regulative role of MALAT1 over miR-124 and the biological function of this axis over growth and invasion of HR-HPV (+) cervical cell are verified, we further explored the downstream target of miR-124. Through prediction in Targetscan 6.2, we identified GRB2 is a highly possible target of miR-124 (Fig. 4a). Therefore, we constructed two

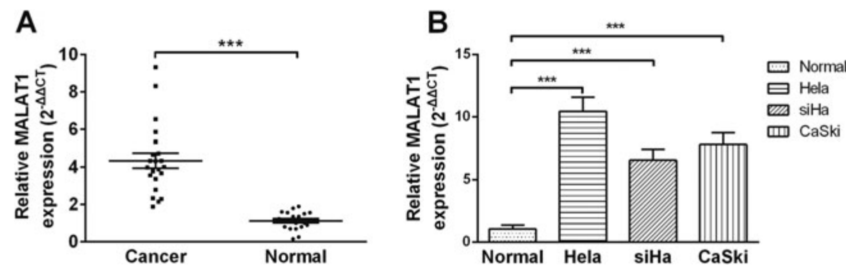

Fig. 1 MALAT1 expression is significantly upregulated in HR-HPV (+) cervical cancer. **a** qRT-PCR analysis of MALAT1 expression in cervical tumor and normal tissues from 22 HR-HPV-positive cervical cancer patients and 22 HR-HPV-negative healthy controls. **b** qRT-PCR analysis of MALAT1 expression in HPV16-positive CaSki and SiHa cells and in HPV18-positive HeLa cells. *$P<0.05$; **$P<0.01$, ***$P<0.001$

dual luciferase reporters to verify the putative binding sites. In both Hela and siHa cells, miR-124 could effectively reduce the relative luciferase activity of wild-type reporter, but not the reporter carrying mutant sequence (Fig. 4b, c). Knockdown of endogenous MALAT1, similar as overexpression of miR-124, could significantly decrease GRB2 expression at both mRNA (Fig. 4d) and protein level (Fig. 4e, f). MALAT1 knockdown and miR-124 overexpression simultaneously had stronger inhibiting effect than MALAT1 knockdown alone (Fig. 4d–f). To further explore the effect of GRB2 over cell growth and invasion, Hela and siHa cells were transfected with GRB2 siRNA (Fig. 4g, h). As expected, knockdown of endogenous

Fig. 2 MALAT1 involves in regulation of cell growth and invasion of HR-HPV (+) cervical cancer cells. **a**, **b** qRT-PCR analysis of MALAT1 expression in Hela (**a**) and siHa cells (**b**) transfected with MALAT1 siRNA. **c**, **d** CCK-8 assay of viability of Hela (**c**) and siHa (**d**) cells 24, 48, and 72 h after transfection. **e** Representative images of invaded Hela and siHa cells in transwell invasion assay. **f** Quantification of invaded Hela and siHa cells showed in **e. g** Representative images of Hela and siHa cells with active caspase-3. **f** Quantification of apoptotic Hela and siHa cells showed in **g**. *$P<0.05$; **$P<0.01$, ***$P<0.001$, *NS* not significant

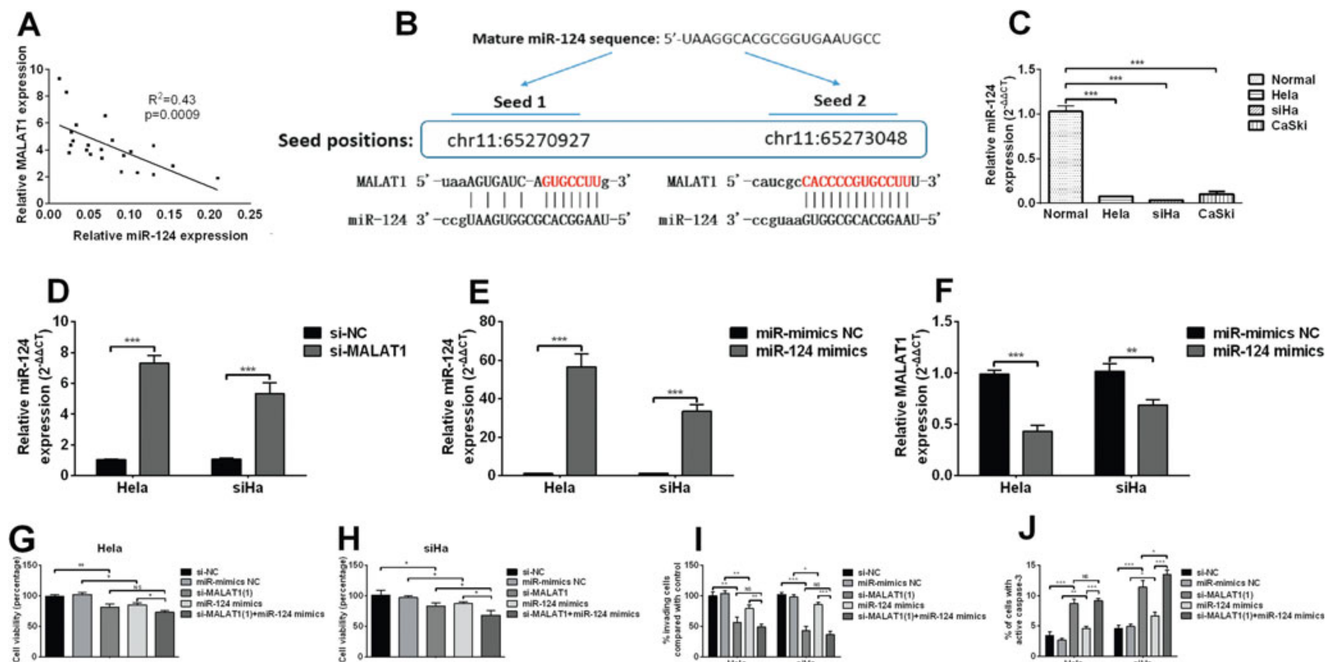

Fig. 3 MALAT1 regulates cell growth and invasion through interacting with miR-124. **a** Linear regression analysis of MALAT1 and miR-124 expression in tumor tissues from 22 HR-HPV (+) cervical cancer patients. **b** Predicted binding sites between MALAT1 and miR-124. **c** qRT-PCR analysis of miR-124 expression in Hela, siHa, and CaSki cells. **d** qRT-PCR analysis of miR-124 expression in Hela and siHa cells 24 h after transfection with MALAT1 siRNA. **e** qRT-PCR analysis of miR-124 expression in Hela and siHa cells 24 h after transfection with miR-124 mimics. **f** qRT-PCR analysis of MALAT1 expression in Hela and siHa cells 24 h after transfection with miR-124 mimics. **g**, **h** CCK-8 assay of viability of Hela (**g**) and siHa (**h**) 48 h after transfection with MALAT1 siRNA and miR-124 mimics alone or in combination. **i** Quantification of transwell analysis of invaded Hela and siHa cells transfected with MALAT siRNA and miR-124 mimics alone or in combination. **j** Quantification of flow cytometry analysis of apoptotic Hela and siHa cells transfected with MALAT1 siRNA and miR-124 mimics alone or in combination. **P*<0.05; ***P*<0.01; ****P*<0.001, *NS* not significant

GRB2 significantly reduced cell invasion (Fig. 4i, j) and increased cell apoptosis (Fig. 4k, l). These results suggest that miR-124 can target GRB2 and regulate its expression, thereby affecting cell invasion and apoptosis.

Discussion

MALAT1 is generally upregulated in several types of cancer and is viewed as an oncogene [18]. In the current study, we also verified higher MALAT1 expression in cancer tissues from patients with HR-HPV (+) cancer. Its oncogenic role has been gradually revealed in different cancers. For example, high MALAT1 expression is associated with high stage, metastasis, and shorter overall survival after radical nephrectomy in patients with renal cell carcinoma [19]. It can also promote brain metastasis of non-small cell lung cancer (NSCLC) by inducing epithelial-mesenchymal transition [20]. In colorectal cancer cells, MALAT1 can promote cancer cell proliferation, migration, and invasion via PRKA kinase anchor protein 9 [21]. In cervical cancer cell CaSki, knockdown of MALAT1 resulted in significantly decreased cell cycle regulation molecules cyclinD1, cyclinE, and CDK6, and thereby increasing the ratio of cells arrested in G1 phase. Therefore, MALAT1 may regulate cell proliferation through the P16INK4A/CDKs/RB pathway [9]. In the present study, we also found that MALAT1 involves in cell growth and invasion of the HR-HPV (+) cancer cells. Knockdown of endogenous MALAT1 significantly reduced cell growth rate and invasion and increased cell apoptosis of Hela and siHa cells. However, what mechanisms are involved in these biological functions of MALAT1 should be further studied.

Recently, a novel regulatory mechanism of RNA, competing endogenous RNAs (ceRNAs) have been proposed. This notion indicates that RNAs can crosstalk with each other through competing shared for miRNAs and thereby modulating the bioavailability of miRNAs on their targets and imposing another level of posttranscriptional regulation [22, 23]. We thus explored the potential of MALAT1 exerting functions through targeting miRNAs. We performed a search for miRNAs that had complementary base pairing with MALAT1. Through our preliminary studies, we found miR-124 is a highly possible target of MALAT1 since it showed a negative expression trend to MALAT1 in the tumor tissues from the patients.

MiR-124 is a tumor suppressor in several types of cancer, including hepatocellular [11], gastric [12], hematological [24], and cervical cancer [13]. In cervical cancer, miR-124 can

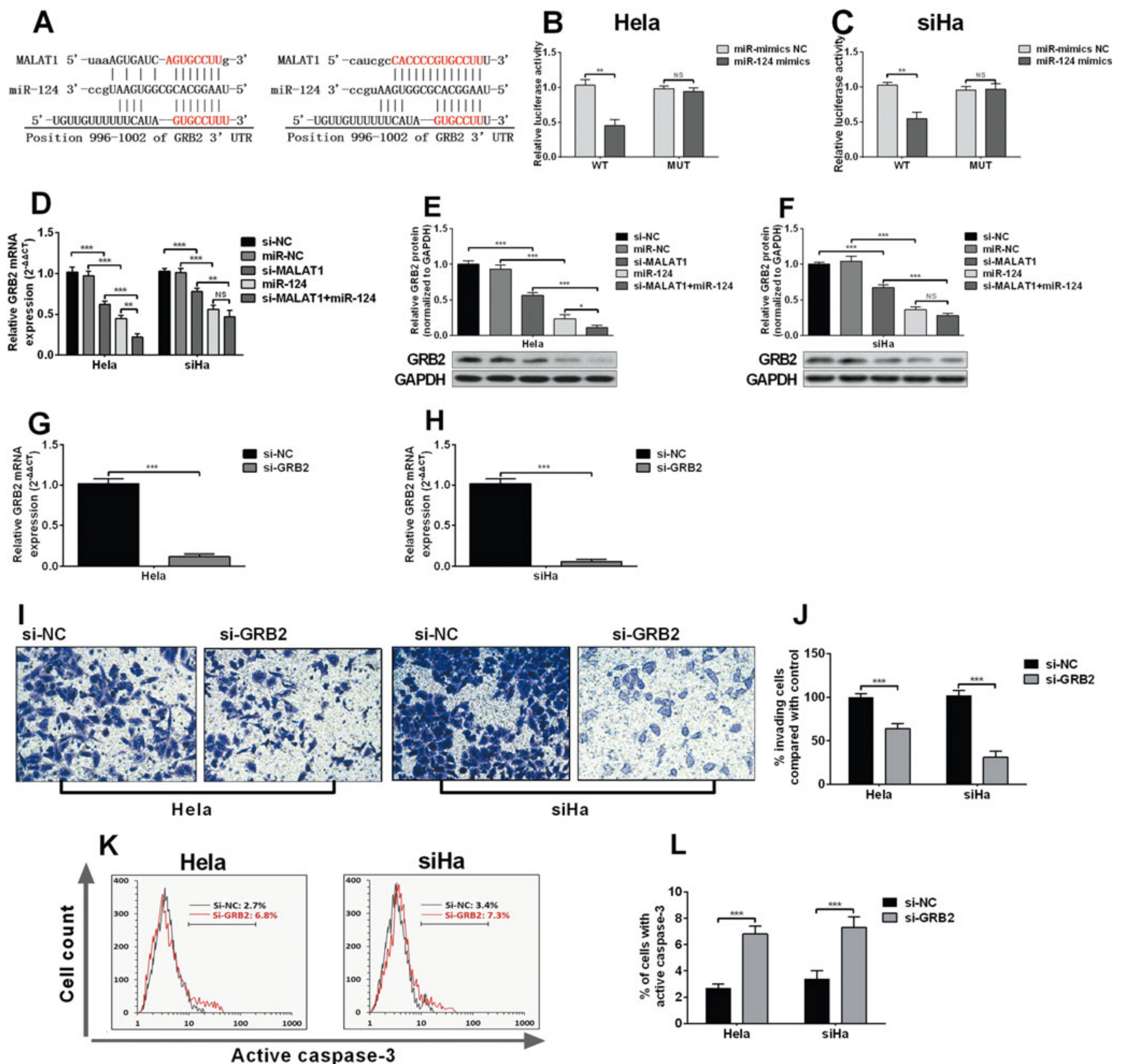

Fig. 4 MiR-124 directly targets GRB2 and regulates its expression, thereby affecting cell invasion and apoptosis. **a** Predicted binding sites among MALAT1, miR-124, and 3′UTR of GRB2 mRNA. **b**, **c** Hela (**b**) and siHa (**c**) cells were co-transfected with either 50 nM miR-124 mimics or NC oligos and 200 ng dual luciferase reporter plasmids carrying either WT or MUT 3′-UTR of GRB2. The relative firefly luciferase activity measured 24 h after transfection and was normalized with renilla luciferase activity. **d** qRT-PCR analysis of GRB2 mRNA expression 48 h after transfection with MALAT1 siRNA and miR-124 mimics alone or in combination. **e**, **f** Western blot analysis of GRB2 expression at protein level in Hela (**e**) and siHa (**f**) cells 48 h after transfection with MALAT1 siRNA and miR-124 mimics alone or in combination. **g**, **h** qRT-PCR analysis of GRB2 mRNA expression 48 h after transfection GRB2 siRNA in Hela (**g**) and siHa (**h**) cells. **i** Representative images of invaded Hela and siHa cells in transwell invasion assay. **j** Quantification of invaded Hela and siHa cells showed in **i.** **k** Representative images of Hela and siHa cells with active caspase-3. **l** Quantification of apoptotic Hela and siHa cells showed in **k**. *$P<0.05$; **$P<0.01$; ***$P<0.001$, *NS* not significant

repress vasculogenic mimicry, migration, and invasion in HeLa and C33A cells by targeting AmotL1 [13]. Ectopic hsa-miR-124 expression in SiHa and CaSki cells can decrease proliferation rates and migratory capacity [15]. Low miR-124 expression in cervical cancer might be a result of multiple causes. Based on available evidences, methylation-mediated silencing and SNP are two important causes [14, 15]. We decided to study whether ceRNA is a mechanism of its down-regulation. Our bioinformatics study showed that MALAT1 has two putative binding sites with miR-124. Knockdown of

MALAT1 increased the expression of miRNA-124, while ectopic expression of miR-124 decreased MALAT1 expression. Therefore, there is a reciprocal interaction between MALAT1 and miR-124. Knockdown of MALAT1 can inhibit cell growth and invasion and increase cell apoptosis. MALAT1 knockdown and miR-124 overexpression simultaneously had some level of enhanced effect. Therefore, our study suggests that MALAT1 may promote tumor development through "spongeing" miRNA-124.

A miRNA usually has multiple targets. Besides amotL1, we tried to explore whether there are other targets of miR-124 in cervical cancer. Growth factor receptor-bound protein 2 also known as GRB2 is an adaptor protein involved in signal transduction/cell communication [25]. HPV-16 infection is a cause of significantly increased GRB2 expression in cervical cancer, of which is involved in enhanced EGFR internalization and mTORC1 activation [26] and KGFR/FGFR2b-mediated epithelial growth [27]. In the present study, we verified a direct interaction between miR-124 and 3′UTR of GRB2. Therefore, MALAT1 can indirectly modulate GRB2 expression via competing miR-124. Knockdown of GRB2 reduced cell invasion and increased cell apoptosis. Due to the length of lncRNA, it is highly possible that one lncRNA can modulate the expression of multiple miRNAs. Therefore, although this study identified the role of MALAT1-miR-124-RBG2 axis in cervical cancer, it is difficult to ascribe the function of an lncRNA or a miRNA to a single target. We cannot preclude the possibility of functional contributions by other MALAT1 or miR-124 targets.

In conclusion, MALAT1 can promote HR-HPV (+) cancer cell growth and invasion at least partially through MALAT1-miR-124-RBG2 axis. This finding might provide some useful evidence about the lncRNA interaction regulatory network in tumorigenesis cervical cancer.

Conflicts of interest None

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