HOTAIR/miR-203/CAV1 Crosstalk Influences Proliferation, Migration, and Invasion in the Breast Cancer Cell

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Abstract: In recent years, malignant breast cancer metastasis has caused a great increase in mortality. Research on the genetic and molecular mechanisms of malignant breast cancer has continued to deepen, and targeted therapy has become the general trend. Among them, competing endogenous RNA (ceRNA)-related molecules have received much attention. Homeobox transcript antisense RNA (HOTAIR) has been reported to function extensively as a ceRNA in breast cancer. Notably, miR-203 and Caveolin 1 (CAV1) have also been found to play a role in breast cancer. However, the relationship between the three remains unclear. In this study, we present a new mechanic through bioinformatics tool and basic experiments: the HOTAIR/miR-203/CAV1 axis, which complemented the role network of HOTAIR as a ceRNA, thus, it will provide a novel potential idea for breast cancer research and therapy.

Keywords: breast cancer; HOTAIR; miR-203; CAV1; ceRNA; invasion; migration

1. Introduction

According to the latest statistics released by the International Agency for Research on Cancer (IARC) in December 2020, breast cancer accounted for 11.7% of new tumor cases in 2020, surpassing lung cancer, which accounted for 11.4% [1]. Breast cancer has become the most common malignant tumor type in the world. Therefore, the prevention, diagnosis, and treatment of breast cancer are urgent. The significance of analyzing breast cancer’s metastasis mechanism and studying the mechanism of malignant proliferation, invasion, and migration of breast cancer cells has reached its peak. Homeobox (HOX) transcript antisense RNA (HOTAIR) was first discovered accidentally in 2007 and is located at 12q13.13, between HOXC11 and HOXC12 on chromosome 12 [2]. Therefore, it is also called intergenic long non-coding RNA (lncRNA), which contains 2158 nucleotides and 6 exons. HOTAIR can regulate gene expression epigenetically in a manner similar to scaffolding and can also interfere with the expression of signaling molecules associated with breast cancer development in breast cancer. Qian L. et al. found that up-regulated HOTAIR in breast tissue can enhance the expression of the DNA damage repair factors and regulate cell cycle and apoptosis [3]. The overexpression of HOTAIR can activate estrogen receptor transcription, inducing breast cancer drug-resistant and promoting cancer cell proliferation [4]. A recent study analyzed the in situ expression of HOTAIR in breast cancer patients and showed that a high expression of HOTAIR in tumor tissue is closely...
related to lymph node metastasis [5]. A population-based case-control study found that HOTAIR is associated with breast cancer risk and prognosis [6]. Based on the important role of HOTAIR in breast cancer progression, a recent review summarized the potentially promising therapeutic function of HOTAIR in breast cancer [7]. However, the underlying mechanism of HOTAIR in breast cancer requires more research.

Caveolin 1 (CAV1) is the gene encoding for caveolin-1. The expression of the CAV1 gene is associated with many human diseases, including pulmonary hypertension, hypertriglyceridemia, and cancer. It has been reported that CAV1 plays a key role in breast cancer cell proliferation, apoptosis, autophagy, invasion, migration, and breast cancer metastasis [8–12]. Another study confirmed the absence of CAV1 gene mutations in human breast cancer [13]. It is only that the expression level of CAV1 is regulated by other factors, and it plays a dual role in inhibition or promotion in the process of breast cancer [14]. In the study of mechanisms in breast cancer, CAV1 has been our target, and we strive to identify the key molecules that act on it.

It has been reported that lncRNAs regulate mRNA expression by binding specific microRNAs (miRNAs) as competing endogenous RNAs (ceRNAs) [15]. Therefore, the physical interaction of ceRNA-miRNAs leads to a complex regulatory network of ceRNA/miRNA/mRNA networks that controls gene expression at the transcriptional, post-transcriptional, and post-translational levels [16,17]. Some studies have found that these interacting ceRNA/miRNA/mRNA networks are involved in many cellular processes and human diseases, including tumorigenesis [18–20]. The ceRNA/mRNA network has also been reported in the study of breast cancer mechanisms. Using a weighted gene co-expression network analysis (WGCNA) algorithm, they found a ceRNA-lncRNA TRPM2 that promotes the proliferation of BRCA cells and inhibits apoptosis through the TRPM2-AS/miR-140-3p/PYCR1 axis [21]. This further inspires us to explore the miRNA that can connect lncRNA HOTAIR and CAV1 and improve the role of the ceRNA/mRNA network in the progression of breast cancer.

In this study, we first analyzed the differential expression of HOTAIR in breast cancer and normal tissues by using database and online messaging tools, screened the possible HOTAIR-binding miRNA, enriched some of the miRNAs, verified the role of miR-203 in cancer, and chose the miR-203 which we are interested in. Then, we analyzed the key protein molecules and their corresponding genes by protein–protein interaction (PPI) and found that CAV1 was included in the PPI network. The dual-luciferase reporter gene, cell proliferation, migration, and invasion in vitro were used to verify the sponge effect of HOTAIR on miR-203 and the regulation of miR-203 on CAV1 expression. Finally, the role of the HOTAIR/miR-203/CAV1 axis in the proliferation, invasion, and migration of the breast cancer cell line MDA-MB-231 was clarified.

2. Results
2.1. High Expression of HOTAIR in Breast Cancer

To reveal the function of HOTAIR in cancer, we first used GEPIA (http://gepia.cancer-pku.cn/detail.php, accessed on 12 March 2022) to analyze the expression of HOTAIR in different cancers. The results showed that, compared with normal tissues, HOTAIR was highly expressed in breast cancer (Figure 1A). Through the UALCAN (http://ualcan.path.uab.edu/index.html, accessed on 12 March 2022) online tool to analyze the TCGA database, the results showed that HOTAIR was significantly overexpressed in luminal breast cancer, HER2-positive breast cancer, and triple-negative breast cancer (TNBC) (Figure 1B). Based on the subclasses, the UALCAN results showed that HOTAIR was overexpressed in breast invasive carcinoma compared with normal tissues (Figure 1C). The Kaplan–Meier curves and log-rank analysis results suggested that patients with a high expression of HOTAIR may have a worse prognosis (Figure 1D).
Figure 1. HOTAIR is highly expressed in breast cancer. Data analysis from GEPIA and UALCAN. (A) Among various cancers, HOTAIR was significantly overexpressed in breast cancer. (B) HOTAIR was significantly overexpressed in luminal breast cancer, HER2-positive breast cancer, and TNBC. (C) Compared with normal tissues, HOTAIR was significantly overexpressed in breast invasive carcinoma. (D) Breast cancer patients with high expression of HOTAIR might have a poorer prognosis. BRCA, breast invasive carcinoma; LUSC, lung squamous cell carcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; TGCT, testicular germ cell tumors; TNBC, triple-negative breast cancer; UCEC, uterine corpus endometrial carcinoma; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; TNBC, triple-negative breast cancer. Values were significantly different compared with the corresponding control value at *p < 0.05.

2.2. Up-Regulation of HOTAIR Promotes the Proliferation, Invasion, and Migration of MDA-MB-231 Cells

The expression of HOTAIR was higher in MDA-MB-231 than in MCF-7 (Figure 2A); thus, we selected a highly invasive breast cancer cell line, MDA-MB-231 (a type of TNBC-cells), for cell transfection in the subsequent experiments. The results of CCK-8 showed that the proliferation rate of down-regulated HOTAIR cells decreased, while the proliferation rate of overexpressed HOTAIR cells increased significantly (Figure 2B). The transwell assay results showed that the ability of migration and invasion in overexpressed HOTAIR cells was significantly increased, while in down-regulated HOTAIR cells, it was decreased (Figure 2C). The wound healing assay results revealed that HOTAIR could strikingly enhance the migration of MDA-MB-231 (Figure 2D). These results all proved that the up-regulation of HOTAIR could promote the proliferation, migration, and invasion of MDA-MB-231 cells.
To study the relation between HOTAIR (as ceRNA) and miR-203 in breast cancer, we predicted the potential miRNAs targets of HOTAIR by using ENCORI (StarBase, https://starbase.sysu.edu.cn/index.php, accessed on 12 March 2022) and DAVID (https://david.ncifcrf.gov/tools.jsp, accessed on 12 March 2022). We exported the data (Table S1) from the two databases. Software Cytoscape 3.0 was used to construct a network map of miRNAs that may bind to HOTAIR (Figure 3A). Of note, miR-203 was one of the targets (Figure 3A). Then, we performed GO functional annotation and pathway enrichment analysis using miEAA 2.0 (https://ccb-compute2.cs.uni-saarland.de/mieaa, accessed on 12 March 2022) to examine the functions of these miRNAs. The GO analysis was conducted at three sublevels: biological process (BP), molecular function (MF) and molecular composition (CC). The results of MF and CC are the same. The results showed that the overexpression of miR-203 was significantly enriched in the regulation of angiogenesis, cell proliferation, and apoptosis, such as angiogenesis, blood vessel morphogenesis, and the positive regulation of the cell population in the BP category (Figure S1); negative regulation of anoikis, wound healing, B-cell lymphoma-2 family protein complex, and the extrinsic...
apoptotic signaling pathway in the MF and CC categories (Figures S2 and S3). KEGG’s cell signaling pathway was also conducted. The miR-203-enriched KEGG pathways were MicroRNAs in cancer, pathways in cancer and Adherens junction (Figure S4). Compared with normal tissues, miR-203 was overexpressed in breast invasive carcinoma through the UALCAN analysis of the TCGA database (Figure 3B). Moreover, miR-203 was significantly overexpressed in luminal breast cancer, HER2-positive breast cancer, and TNBC (Figure 3C). The Kaplan–Meier analysis suggested that patients with a high expression of miR-203 may have a worse prognosis (Figure 3D). From the above analysis, it can be concluded that miR-203 plays an important role in the proliferation, survival, migration, and invasion of cells, especially cancer cells. Notably, these roles of miR-203 are similar to HOTAIR. Therefore, we have reason to believe that HOTAIR is one of the ceRNAs of miR-203. To further verify the binding of HOTAIR-miR-203 in cancer cells, we constructed the wild-type plasmid HOTAIR-wt of HOTAIR that contains the complementary sequence of miR-203, and the mutant plasmid HOTAIR-mu with the mutation of the complementary binding sequence (Figure 3E). Compared with the NC group, the luciferase activity of the miR-203 mimics + HOTAIR-wt group was decreased, while the miR-203 mimics + HOTAIR-mu group luciferase activity changes were less pronounced (Figure 3F), showing the existence of binding between HOTAIR and miR-203.

2.4. HOTAIR Reverses the Effect of miR-203 on MDA-MB-231 Cells in Proliferation, Invasion, and Migration

To further verify the role of HOTAIR/miR-203 in breast cancer progression, we trans-fected the MDA-MB-231 cells with the HOTAIR plasmid. The RT-qPCR results showed that the expression level of miR-203 was significantly increased when HOTAIR was down-regulated, and the expression level of miR-203 was significantly decreased when HOTAIR was up-regulated (Figure 4A). The results of the CCK assay showed that inhibiting the expression of miR-203 would promote the proliferation of MDA-MB-231 cells, and down-regulating HOTAIR reversed the effect of the miR-203 inhibitor on the proliferation of MDA-MB-231 cells (Figure 4B). The transwell assays showed that the inhibition of miR-203 expression promoted the migration and invasion of MDA-MB-231 cells, and the down-regulation of HOTAIR reversed the effect of the miR-203 inhibitor on the promotion of MDA-MB-231 cell migration and invasion (Figure 4C). The wound healing assay results showed that inhibiting the expression of miR-203 would promote the migration of MDA-MB-231 cells, and down-regulating HOTAIR reversed the effect of the miR-203 inhibitor on promoting the migration of MDA-MB-231 cells (Figure 4D). The above results all proved that HOTAIR/miR-203 could regulate the proliferation, migration, and invasion of MDA-MB-231 cells.

2.5. miR-203 Binds to CAV1 and Inhibits the Expression of CAV1

The last link is missing in our constructed ceRNA/miRNA/mRNA axis. Therefore, GO (GO-Biological Process) in GeneCards (https://www.genecards.org/, accessed on 12 March 2022) for the miR-203 involved in gene silencing by miRNA was analyzed (GO:0035195, Figure 5A). In addition, we used the online tool, miEAA 2.0, to enrich the target genes corresponding to the miRNAs (including miR-203) that may be bound by HOTAIR, and screened all the target genes containing miR-203 in the enrichment (Table S2). Then, STRING (https://cn.string-db.org/, accessed on 12 March 2022) was used to make a network map of the interaction (PPI) between the protein encoded by the target gene and the protein encoded by CAV1 (Figure 5B). Notably, CAV1 connected five edges as one of the nodes (Figure 5B), indicating that CAV1 may be in the regulatory network of miR-203. Therefore, we transfected the MDA-MB-231 cells with miR-203 mimics and miR-203 inhibitors to verify the effect of miR-203 on CAV1 expression. The Western blot results showed that when the expression of miR-203 was down-regulated, the expression level of caveolin-1 was significantly increased. When the expression of miR-203 was up-regulated, the expression level of caveolin-1 was decreased (Figure 5C). To further verify whether miR-203 binds to
CAV1, we constructed the wild-type plasmid CAV1-wt that contains the complementary sequence of miR-203, and mutant plasmid CAV1-mu, with a mutation of the complementary binding sequence (Figure 5D). The experimental results obtained by the dual-luciferase reporter gene assay are shown in Figure 5E. Compared with the NC group, transfection miR-203 could significantly down-regulate the luciferase activity of the CAV1-wt group. However, transfection of miR-203 could not significantly change the luciferase activity of the CAV1-mu group, which proved that miR-203 could directly bind to CAV1. Therefore, miR-203 could interact with CAV1, and the overexpression of miR-203 could inhibit the expression of CAV1.

Figure 3. miR-203 functions in cancer, and HOTAIR acts as a molecular sponge for miR-203. (A) Data of possible HOTAIR-bound miRNAs were derived by comprehensive analyses of ENCORI and DAVID. The HOTAIR-miRNA network map was made by Cytoscape3.0 software. (B) Compared with normal tissues, miR-203 was overexpressed in breast invasive carcinoma through the UALCAN analysis of the TCGA database. (C) miR-203 was significantly overexpressed in luminal breast cancer, HER2-positive breast cancer, and TNBC. (D) Breast cancer patients with high expression of miR-203 might have a poorer prognosis. (E) Putative complementary sites between HOTAIR and miR-203. Mutations were generated in the HOTAIR nucleotides complementary to miR-203. (F) Fluorescence expression was detected by the dual-luciferase reporter gene. Compared with the NC group, the luciferase activity of the miR-203 mimics/HOTAIR-wt group decreased, while the luciferase activity of the miR-203 mimics/HOTAIR-mu group did not change significantly (p > 0.05). Values were significantly different compared with the corresponding control value at *** p < 0.001. ns > 0.05. K means 1000. IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; TNBC, triple-negative breast cancer.
Figure 4. HOTAIR reverses the effect of miR-203 on MDA-MB-231 cells in proliferation, invasion, and migration. (A) RT-qPCR results showed that compared with the NC group, the expression level of miR-203 was significantly increased after down-regulation of HOTAIR, while the expression level of miR-203 was extremely significantly decreased after up-regulation of HOTAIR. (B) CCK-8 results showed that after 48 h and 72 h, compared with the NC group, the cell proliferation rate in the miR-203 inhibitor group was significantly higher than that in the miR-203 mimics group; compared with the miR-203 inhibitor group, the rate of cell proliferation decreased in the inhibitor + siHOTAIR group. (C) The transwell invasion and migration assay results showed that, compared with the NC group, the number of cell invasions and migrations in the miR-203 inhibitor group was significantly increased. In the inhibitor + siHOTAIR group, the number of cell invasions and migrations significantly decreased. (D) The results of the wound healing assay showed that the migration area in miR-203 inhibitor cells was significantly increased, while in the inhibitor + siHOTAIR group, the migration ability of cells was decreased. Values were significantly different compared with the corresponding control value at *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. 
The wound healing assay showed that the overexpression of CAV1 could promote the proliferation, invasion, and migration of MDA-MB-231 cells. The results of CCK-8 showed that the overexpression of CAV1 could promote the proliferation of MDA-MB-231 cells, and up-regulation of the expression of miR-203 could reverse the promoting effect of the overexpression of CAV1 on the proliferation rate of MDA-MB-231 cells (Figure 6A). The transwell assay showed that the overexpression of CAV1 could promote the migration and invasion of MDA-MB-231 cells, and up-regulation of miR-203 reversed the promoting effect of CAV1 overexpression on the migration and invasion ability of MDA-MB-231 cells (Figure 6B). The wound healing assay showed that the overexpression of CAV1 could promote the migration of MDA-MB-231 cells, and up-regulation of miR-203 reversed the promotion of CAV1 overexpression on the migration ability of MDA-MB-231 cells (Figure 6C). The above in vitro experiments all proved that the overexpression of CAV1 could promote the progression of breast cancer cells, and the interaction of miR-203/CAV1 could regulate the proliferation, invasion, and migration of MDA-MB-231 cells.

**Figure 5.** miR-203 binds to CAV1 and inhibits the expression of CAV1. (A) Gene Ontology (GO-Biological Process) for miR-203 involved in gene silencing (GO: 0035195). (B) PPI network diagram between miR-203 target gene-encoded proteins and CAV1-encoded proteins derived from STRING. (C) Western blot detection showed that the expression level of caveolin-1 was significantly increased in the miR-203 down-regulated group. (D) Putative complementary sites between miR-203 and CAV1. Mutations were generated in the CAV1 nucleotides complementary to miR-203. (E) Dual-luciferase reporter gene detection of the interaction between miR-203 and CAV1. Compared with NC, the expression of luciferase in mimics + CAV1-wt decreased, and the expression of luciferase in mimics + CAV1-mu was unchanged. Values were significantly different compared with the corresponding control value at *p < 0.05, ***p < 0.001, and ****p < 0.0001. ns > 0.05.

### 2.6. miR-203 Restrains the Function of CAV1 on MDA-MB-231 Cells in Proliferation, Invasion, and Migration

We further investigated, in vitro, that miR-203/CAV1 regulated the proliferation, invasion, and migration of MDA-MB-231 cells. The results of CCK-8 showed that the overexpression of CAV1 could promote the proliferation of MDA-MB-231 cells, and up-regulation of the expression of miR-203 could reverse the promoting effect of the overexpression of CAV1 on the proliferation rate of MDA-MB-231 cells (Figure 6A). The transwell assay showed that the overexpression of CAV1 could promote the migration and invasion of MDA-MB-231 cells, and up-regulation of miR-203 reversed the promoting effect of CAV1 overexpression on the migration and invasion ability of MDA-MB-231 cells (Figure 6B). The wound healing assay showed that the overexpression of CAV1 could promote the migration of MDA-MB-231 cells, and up-regulation of miR-203 reversed the promotion of CAV1 overexpression on the migration ability of MDA-MB-231 cells (Figure 6C). The above in vitro experiments all proved that the overexpression of CAV1 could promote the progression of breast cancer cells, and the interaction of miR-203/CAV1 could regulate the proliferation, invasion, and migration of MDA-MB-231 cells.
Figure 6. miR-203 inhibits the effect of CAV1 on MDA-MB-231 cells in proliferation, invasion, and migration. (A) CCK-8 results showed that the down-regulation of CAV1 significantly reduced the cell proliferation rate after 48 h and 72 h. Compared with the oeCAV1 group, the proliferation rate of the cells in the co-transfected miR-203 (oeCAV1 + mimics) group decreased. (B) The transwell invasion and migration assay results showed that the number of MDA-MB-231 cells invading and migrating in the oeCAV1 group increased. The number of cell invasions and migrations in the oeCAV1 + mimics group was lower than that in the oeCAV1 group. (C) The wound healing assay showed that the migration area of cells in the oeCAV1 group increased. The migration area of cells in the oeCAV1 + mimics group was smaller than that in the oeCAV1 group. Values were significantly different compared with the corresponding control value at *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

3. Discussion

LncRNA is a type of non-coding RNA, exerting as ceRNA. In recent years, the role of the ceRNA networks in cancer has been extensively studied, linking the functions of protein-coding mRNAs to those of non-coding RNAs [17]. miRNAs are also non-coding RNAs with a length of about 22 nt and play a crucial role in regulating gene expression [22]. They exert regulatory effects by binding to the 3′-untranslated region of target mRNAs, leading to mRNA degradation or silencing in a sequence-specific manner [22]. Given that any transcript containing miRNA-response elements could theoretically function as a ceRNA, they likely represent a broad form of post-transcriptional regulation of gene expression in physiology and pathology. HOTAIR is the first lncRNA discovered to have trans-regulatory roles [2]. Since the discovery of HOTAIR, multiple studies have elucidated its critical role in tumor growth, apoptosis, invasion, metastasis, tumor stem cell differentiation, and drug resistance. Notably, HOTAIR has been reported to function extensively as a ceRNA in breast cancer. Zhao W. et al. found that HOTAIR affected MDA-MB-231 cell growth
and apoptosis through the miR-20a-5p/HMGA2 pathway [23]. Wu D. et al. found that the HOTAIR/miR-129-5p/Fzd7 axis could accelerate breast cancer progression [24]. In addition, HOTAIR could increase radioresistance in breast cancer by promoting HSPA1A expression through binding to miR-449b-5p in MDA-MB-231 cells [25].

Breast cancer studies are limited to a few cell lines, with MCF-7, T47D, and MDA-MB-231 accounting for the majority of studies [26]. Notably, MDA-MB-231 is a highly aggressive and poorly differentiated triple-negative breast cancer cell line [27]. In recent years, articles on tumor metastasis, drug resistance, and treatment based on MDA-MB-231 have been emerging. Research on HOTAIR has been studied in MDA-MB-231. It is of interest to continue to study MDA-MB-231 to construct a more complete network of actions of HOTAIR. Additionally, in our study, we found that the level of HOTAIR was higher in MDA-MB-231 than in MCF-7 (Figure 2A). In addition, the results (Figure 1C) showed that HOTAIR was higher in TNBC, as known to all, that MDA-MB-231 is a type of TNBC cell. Therefore, we chose the MDA-MB-231 cell line. In this study, we demonstrated that the overexpression of HOTAIR significantly promoted the proliferation, invasion, and migration in MDA-MB-231 cells, which was consistent with the predictions using bioinformatics analysis and previous research.

Further, miR-203, a member of the miRNA family, has been confirmed to be involved in regulating the proliferation, differentiation, metastasis, invasion, and apoptosis of tumor cells [28,29]. Currently, only three studies have addressed the role of miR-203’s link with HOTAIR in cancer [30–32]. The connection between the two deserves further investigation. In this study, we predicted the role of miR-203 in biological and cancer processes through database information. Notably, HOTAIR can compete for binding to miR-203, which is a molecular sponge of miR-203. The dual-luciferase reporter gene assay showed the combination of the two. Furthermore, we demonstrated that the expression of miR-203 was significantly decreased after the overexpression of HOTAIR. The inhibition of HOTAIR reversed the promotion of breast cancer cell progression induced by miR-203 down-expression. That is, HOTAIR could reverse the inhibitory effect of miR-203 on the proliferation, invasion, and migration of MDA-MB-231 cells. Notably, through the TCGA database, we analyzed the expression of miR-203 in breast cancer and found that the expression of miR-203 was different in different breast subtypes. However, its expression was higher than that in normal tissues. In addition to our results, other existing experimental data showed that miRNA was used as a tumor suppressor, such as miR-203, identified as a transcript of matrix stiffness inhibition, which was negatively correlated with the malignant proliferation of breast epithelium [33]. Further, miR-203 also inhibited the migration ability of MDA-MB-231 cells by targeting PRKCG [34]. There are also studies that directly conclude that LncRNA DLG1-AS1 may promote cancer cell proliferation in TNBC by down-regulating the tumor suppressor, miR-203 [35]. This suggests that the role of miR-203 in breast cancer is intriguing, and the mechanism behind its expression, regulation by other molecules, and tumor inhibition is worthy of further exploration.

Recently, CAV1 has been found to be actively involved in human tumor progression [36]. On the one hand, high expression of CAV1 has been reported to drive tumorigenesis by inhibiting apoptosis and promoting anchorage-independent growth, drug resistance, and metastasis [37–39]. For example, CAV1 expression in pancreatic cancer cells was found to be positively correlated with cachectic states [40]. On the other hand, CAV1 acts as a tumor suppressor in some cases, as its low expression favors tumor progression. For instance, Geletu et al. found that CAV1 down-regulation accelerated the proliferation of lung cancer cells via the Cadherin-11/Stat3 axis [41]. Notably, in recent studies, the dual role of CAV1 is evident in breast cancer progression. Loss of stromal CAV1 in human breast cancer was associated with a poor clinical outcome [42]. CAV1 could suppress breast cancer development [43]. However, Dong et al. found that a high expression of CAV1 promoted the migration and invasion of breast cancer cells, supporting its oncogenic role [9]. The dual role of CAV1 in breast cancer deserves further exploration. Moreover, the regulation of CAV1 expression also remains poorly understood. Therefore, our team has been devoted
to the related research of CAV1, trying to elucidate the mechanism of CAV1 in breast cancer progression. Note, however, that the role of CAV1 in association with miR-203 in cancer has only been elucidated in renal cell carcinoma [44]. In this study, the down-regulation of CAV1 expression attenuated the proliferation, invasion, and migration of the breast cancer cell MDA-MB-231. We used bioinformatics technology to find the miR-203 molecule that can affect the expression of CAV1. The binding of CAV1 and miR-203 was further verified by basic experiments. Moreover, the overexpression of miR-203 significantly down-regulated CAV1’s expression and reversed the promotion effect of the MDA-MB-231 cells’ progression induced by CAV1.

4. Materials and Methods

4.1. Database Search

Gene Expression Profiling Interactive Analysis (GEPIA; http://gepia.cancer-pku.cn/detail.php, accessed on 12 March 2022) [45] was used to analyze the expression of HOTAIR in invasive breast cancer. UALCAN (http://ualcan.path.uab.edu/index.html, accessed on 12 March 2022) was used to analyze the differential expression of HOTAIR and miR-203 in breast cancer subtypes and survival analysis in the TCGA database [46]. The ENCORI (StarBase, https://starbase.sysu.edu.cn/index.php, accessed on 12 March 2022) database [47] and DAVID (https://david.ncifcrf.gov/tools.jsp, accessed on 12 March 2022) database [48] were used to analyze the miRNAs interacting with HOTAIR. Cytoscape (3.9.0) was used to draw the HOTAIR-miRNA network map and select the key molecules on the network map. Gene Ontology/Kyoto Encyclopedia of Genes and Genomes (GO/KEGG) analysis on the miRNA set was performed by using the miRNA enrichment analysis and annotation tool, miEAA 2.0, online (https://ccb-compute2.cs.uni-saarland.de/mieaa, accessed on 12 March 2022) [49], and GeneCards (https://www.genecards.org/, accessed on 12 March 2022). Then, enrich the related target genes of miR-203, and use the STRING (https://cn.string-db.org/, accessed on 12 March 2022) database [50] for the proteins encoded by the target genes to export the related protein–protein interaction (PPI) network. Finally, combined with the CAV1, we focused on and analyzed whether it has a role with the above proteins.

4.2. Cell Culture

The human breast cancer cell line (MDA-MB-231and MCF-7) was purchased from Shanghai Cell Bank, the Chinese Academy of Sciences. The human embryonic kidney cell line (HEK-293T) was provided by Shanghai Hanheng Biotechnology Co., Ltd., Shanghai, China. The cells were cultured in DMEM (high glucose) (Hyclone, Logan, UT, USA), supplemented with 10% fetal bovine serum (FBS) (BI, KibbutzBeit Haemek, Israel) and 1% penicillin-streptomycin (Solarbio, Beijing, China) in a cultured environment of 37 °C constant temperature, 5% CO2 incubator.

4.3. Cell Transfection

Before transfection, 3.5 × 10^5 cells were seeded in each well in a six-well plate. When the cells grew to about 70% of the bottom of the culture dish, Lipofectamine 3000 (Thermo Fisher, 81 Wyman Street, Waltham, MA, USA) and Opti-MEM serum-free medium (Gibco, Grand Island, NY, USA) were used for HOTAIR overexpression plasmid (HOTAIR homo pcDNA3.1, oeHOTAIR). Further, siRNA (siRNA 536, siHOTAIR), miRNA mimics (miR-203 mimics), miRNA inhibitor (mir-203 inhibitor), CAV1 overexpression plasmid (CAV1 homo pcDNA3.1, oeCAV1), siRNA (siRNA710, siCAV1), negative control (NC, down-regulated NC is siCON, overexpressed NC is oeCON) were prepared as a transfection complex and added to six wells. The cells were transfected in the plate. The above pcDNA and siRNA were constructed by Suzhou Gene, and the cells were collected after 24 h to extract total RNA to determine the transfection efficiency by qPCR. The sequences are shown in Table S3.
4.4. RNA Extraction and Quantitative Real-Time PCR (RT-qPCR)

Total RNA was isolated from the cells 24 h post-transfection after RNA quantification according to the kit instructions (Omega, Madison, WI, USA), and the reaction system was prepared according to the instructions. RNA (2–5 μg per system) was reverse transcribed into cDNA by using the PerfectStart™ Green qPCR SuperMIX (TransGen Biotech, Beijing, China) and StepOne™ qRT PCR machine (ABI, Waltham, MA, USA) real-time device under the following conditions: 42 °C for 30 min, 85 °C for 5 s. The qPCR system was prepared according to the instructions of the TransStart® Tip Green qPCR SuperMix kit (TransGen Biotech, Beijing, China). The qPCR was performed under the following conditions: 94 °C for 30 s, 40 cycles of 94 °C for 5 s, and 62 °C for 30 s. All results were normalized to U6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative expression level of miRNA was detected by the $-2^{\Delta\Delta C_t}$ method. All primer pairs were synthesized by Sangon (Shanghai, China), and the sequences are shown in Table S4.

4.5. Cell Proliferation Assay

The Cell Counting Kit-8 (CCK-8) (TransGen Biotech, Beijing, China) was used to detect the proliferation of MDA-MB-231, and the cells in the logarithmic growth phase that had been transfected in the early stage were used to inoculate 1000 cells per well in a 96-well plate. More than 4 duplicate wells were set in the group, and the zero-adjustment group only contained medium and CCK. Three 96-well plates were set for 24 h, 48 h, and 72 h. The cells were treated for the first 2 h, washed three times with sterile PBS, and then 90 μL of culture medium and 10 μL of CCK reagent were added to each well. Incubate in the incubator for 1 h, use a microplate reader to measure the OD value of each well at 450 nm and record, and the CCK value is the value obtained by subtracting the blank well from the measured value.

4.6. Cell Migration Assay

The wound healing assay, as well as the transwell assay, were used to assess cell migration. For the wound healing assay: cells were seeded into triplicate wells of a 6-well plate and cultured to 30–50% confluence, and then a 20 μL pipette tip was used to create artificial scratches. Cell images were collected under a microscope at 0 h, 24 h, and 48 h, and the area of the scratched area was measured by software to calculate the migration rate. For the transwell assay: 24 h after transfection, 1 × 10^5 cells were inoculated in a 200 μL serum-free medium in the upper Transwell chamber. A total of 500 μL complete medium containing 10% FBS was added into the lower chamber as a chemoattractant. After 48 h of incubation, we used a cotton swab to remove non-invasive cells manually. Subsequently, the cells were fixed in 4% paraformaldehyde for 30 min, stained with crystal violet for 20 min, and then counted under a microscope.

4.7. Cell Invasion Assay

A Transwell chamber (Corning, Corning, NY, USA) with a Matrigel matrix (Corning, Corning, NY, USA) was used to measure the invasive ability of cells. Before cell plating, the Matrigel matrix was prepared with serum-free DMEM at 200 μg/mL, and 100 μL was evenly spread in each chamber. After clotting, 1 × 10^5 serum-free cultured cells were seeded. An amount of 500 μL complete medium containing 10% FBS was added into the lower chamber as a chemoattractant. After 48 h of incubation, we used a cotton swab to remove non-invasive cells manually. Subsequently, the cells were fixed in 4% paraformaldehyde for 30 min, stained with crystal violet for 20 min, and then counted under a microscope.

4.8. Dual-Luciferase Reporter Assay

The HEK-293T cells were seeded in 96 wells of human embryonic kidney cells, and the density reached 50–70%. An amount of 10 μL DMEM, 0.16 μg HOTAIR/CAV1 target plasmid (HanBIO, Shanghai, China), and 5 pmol miR-203 mimics/negative control (NC) were mixed. After thorough mixing (Solution A), let this stand for 5 min at room temperature.
Mix 10 µL DMEM with 0.3 µL transfection reagent (HanBIO, Shanghai, China, 0.8 mg/mL) thoroughly (Solution B), and let this stand for 5 min at room temperature. Mix solution A and solution B well and leave them at room temperature for 20 min. Add the transfection mixture to the cell culture medium and mix at 37 °C, 5% CO₂. After 6 h of transfection, the fresh medium was changed, and 48 h after transfection, the cells were collected with a kit (Promega Dual-Luciferase, Madison, WI, USA) to measure the luminescence value of the reporter gene.

4.9. Western Blot

Forty-eight hours after the transfection of MDA-MB-231 cells, cell lysates were collected using 100 µL of RIPA lysate (Applygen, Beijing, China) and 10 µL of PMSF per well of a six-well plate. The protein concentration of cell lysates was measured with a BCA protein assay kit (Applygen, Beijing, China). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to nitrocellulose (PVDF) (Millipore, Boston, MA, USA) membranes for Western blotting. The membrane was blocked with 5% nonfat dry milk (BD, Franklin, LA, USA) for 2 h, and then diluted with primary antibody caveolin-1 (Affinity, Shanghai, China, 1:1000). β-actin (ZSGB-Bio, Beijing, China, 1:1000) was used as an internal reference. After overnight incubation, the membranes were washed 3 times with TBST (supplemented with 0.1% Tween 20) and incubated with HPR-labeled goat anti-rabbit IgG (ZSGB-Bio, Beijing, China, 1:1000) dilution for 2 h at room temperature. After washing with TBST, it was reacted with a luminescent solution (TransGen Biotech, Beijing, China) and developed under a gel imaging system.

4.10. Statistical Analysis

We statistically analyzed and calculated the mean ± standard deviation (X ± SD). The charts and experimental data analyzed were created by using GraphPad Primer 8. A student’s t-test was used to analyze the differences between two groups, and one-way analysis of variance (ANOVA) was used to analyze the comparison among multiple groups. A p < 0.05 was defined as the differences that were statistically significant. Further, ns represented no statistically significant difference between data. Image Pro Plus v.6.0 was used to analyze the wound healing and transwell assays. ImageJ software (1.8.0.112) was used to analyze the results from the Western blots.

5. Conclusions

We successfully constructed the HOTAIR/miR-203/CAV1 network and explored the regulatory mechanism of ceRNA-miRNA-mRNA in this network for MDA-MB-231 cells. If only the overexpression of HOTAIR or overexpression of CAV1, or the inhibition of the expression of miR-203 is considered, the proliferation, invasion, and migration of breast cancer MDA-MB-231 cells can be significantly improved. Notably, in our HOTAIR/miR-203/CAV1 network, HOTAIR competes for binding to miR-203, which, in turn, silences CAV1 expression. Therefore, we can boldly propose that, once the expression of HOTAIR is extremely low, the resistance to miR-203 expression is successfully reduced. Then, a large amount of miR-203 is expressed, which, in turn, blocks the production of a large amount of CAV1, thereby successfully inhibiting the progression of breast cancer cells (Figure 7). As our initial validation demonstrates, knockdown of HOTAIR inhibited the proliferation, invasion, and migration of breast cancer cells.
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We successfully constructed the HOTAIR/miR-203/CAV1 network and explored the regulatory mechanism of ceRNA-miRNA-mRNA in this network for MDA-MB-231 cells. If only the overexpression of HOTAIR or overexpression of CAV1, or the inhibition of the expression of miR-203 is considered, the proliferation, invasion, and migration of breast cancer MDA-MB-231 cells can be significantly improved. Notably, in our HOTAIR/miR-203/CAV1 network, HOTAIR competes for binding to miR-203, which, in turn, silences CAV1 expression. Therefore, we can boldly propose that, once the expression of HOTAIR is extremely low, the resistance to miR-203 expression is successfully reduced. Then, a large amount of miR-203 is expressed, which, in turn, blocks the production of a large amount of CAV1, thereby successfully inhibiting the progression of breast cancer cells (Figure 7). As our initial validation demonstrates, knockdown of HOTAIR inhibited the proliferation, invasion, and migration of breast cancer cells.

Although the HOTAIR/miR-203/CAV1 network is of great significance to the progression of breast cancer in MDA-MB-231, what other regulators are involved? The anti-tumor properties of miR-203 and the mechanism of differential expression of related molecules in invasive and non-invasive breast cancer cells deserve further study. However, can these relevant molecules be used as targets for inhibiting breast cancer metastasis? It deserves more exploration. In the future, we will fully decipher this ceRNA/mRNA axis and provide new ideas, directions, and an experimental basis for breast cancer diagnosis, targeted therapy, and prognosis assessment.

Figure 7. HOTAIR/miR-203/CAV1 crosstalk influences proliferation, migration, and invasion in breast cancer cells. In breast cancer cells, the highly expressed HOTAIR might act as a ceRNA to bind with miR-203, which increases the expression of CAV1, thereby promoting the proliferation, invasion, and migration of breast cancer cells. The figure was constructed with BioRender (https://biorender.com/, accessed on 11 May 2022).
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