Breast Cancer: Targets and Therapy

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ORIGINAL RESEARCH

Hsa_circ_0000520 Promotes Invasion and Metastasis of Breast Cancer Cells by Targeting HSP90AA1

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Background: Breast cancer (BC) ranks among the predominant malignancies in females globally. Growing research indicates that circular RNAs (circRNAs) exert important regulatory functions in multiple cancers. Nevertheless, the biological functions and underlying molecular mechanisms of numerous circRNAs in BC remain largely unelucidated.

Methods: Firstly, based on existing database and our previous research, hsa_circ_0000520 has been found to be closely associated with BC and has potential for clinical application. Then, its circular nature was confirmed. The expression in BC cells and tumor tissues was explored by quantitative real-time polymerase chain reaction (qRT-PCR). The biological effects on BC cell growth, invasion and metastasis were verified by vitro experiments. To clarify the molecular mechanism, Chromatin Isolation by RNA Purification (ChIRP) assay coupled with mass spectrometry (MS), RNA immunoprecipitation (RIP), and Western blotting were used to identify and validate interacting proteins. Rescue experiments were conducted using the HSP90AA1 inhibitor tanespimycin. Associations between hsa_circ_0000520 expression and clinicopathological characteristics were analyzed.

Results: Hsa_circ_0000520 was confirmed to be a stable circular RNA predominantly localized in the cytoplasm. Functionally, it served as an oncogene in BC and enhanced the capacities of BC cells to invade and metastasize. Mechanistically, heat shock protein 90 alpha (also termed HSP90AA1) was identified as a key interacting parter of it. Inhibition of HSP90AA1 abrogated the pro-metastatic effects of hsa_circ_0000520 but did not affect its pro-proliferative role, indicating a specific cooperation in driving invasion and metastasis. Clinically, it was significantly increased in BC cells, tumor tissues, and serum samples. Importantly, its elevated expression correlates with advanced clinical stage, specific molecular subtypes, and poor prognosis in BC patients.

Conclusion: Our research results revealed an unrecognized regulatory axis, in which hsa_circ_0000520 facilitates BC cells progression by coordinating with HSP90AA1, highlighting hsa_circ_0000520 could be a promising diagnostic indicator and potential treatment target for BC.

Keywords: hsa_circ_0000520, HSP90AA1, breast cancer, invasion, metastasis

Introduction

Breast cancer (BC) is among the most common malignancies affecting women and continues to be a major cause of cancer-related death globally.[1] Despite notable advances have been made in BC diagnosis and therapy, morbidity and mortality continue to be high owing to the high rates of recurrence and metastatic spread.[2,3] Early detection and intervention are vital for reducing negative events related to BC.[4] At present, in the diagnosis and treatment of BC, common indicators such as CA153 have problems with unsatisfactory sensitivity and specificity, making it impossible to achieve early diagnosis and difficult to make precise predictions and prognoses. Thus, identifying reliable biomarkers and therapeutic targets for BC is of critical importance.

Over the past decades, cancer therapy has evolved from non-specific cytotoxic agents to precision oncology approaches that target molecular drivers of tumorigenesis.[5] The discovery and validation of reliable biomarkers have

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been central to this paradigm shift, enabling early detection, patient stratification, and personalized treatment strategies. Within this context, non-coding RNAs have emerged as promising biomarker candidates due to their tissue specificity and stability. Accumulating evidences have found that circRNAs are a non-coding RNAs expressed across diverse cell types and show promising application prospects. CircRNAs are characterised by a closed continuous loop structure lacking 5′ cap and 3′ tail, are stable, resistant to nuclease degradation, and are involved in the regulation of various physiological and pathological processes.[6] Therefore, it has inherent advantages in terms of sample collection and preservation in clinical testing. CircRNAs exert their functions via diverse mechanisms, including acting as miRNA sponges, protein decoys, RNA-binding protein scaffolds, splicing and translation, among others.[7,8] Nonetheless, the study of interactions between circRNAs and proteins in human cancers has been limited. Because of their high structural stability, circRNAs can be easily found in serum samples and exosomes, underscoring their promise for use as diagnostic indicators.[9–11] Earlier studies have emphasized the importance of using circRNA for diagnostic purposes in breast,[12,13] colorectal,[14,15] bladder,[16,17] gastric[18,19] and lung cancers.[20] In breast cancer, circRNA_0006528,[21] circRNF20,[22] circFBXW7, circABCB10 and circ0103552[23] have been demonstrated to play critical roles in managing cellular proliferation, migratory capacity, metabolic processes, and tumor advancement, and their expression is connected to elevated TNM stages and prognosis. This research is only a small glimpse of the larger context. Further studies are necessary to explore the functional circRNAs and their possible mechanisms and clinical applications in breast cancer.

Heat shock proteins (HSPs) are a group of proteins that are widely present and highly conserved in cells, and play a crucial role in maintaining cellular functions. HSP90 is an extremely important type within the HSPs family, being an ATP-dependent protein with ATPase activity. Among them, HSP90AA1 is the most abundant subtype of HSP90. HSP90AA1, known as a cancer chaperone,[24,25] is a molecular chaperone for a wide range of proteins that modulate key signaling cascades involved in cell proliferation, differentiation, invasive behavior, metastatic spread, programmed cell death, angiogenic activity, and overall tumorigenesis.[26–28] Earlier researches have confirmed that HSP90AA1 enhances the migratory capacity of BC cells and facilitates metastatic progression.[29–31] HSP90AA1 is markedly upregulated in numerous cancers, including breast cancer, and is typically indicative of a worse prognosis.[32] Inhibition of HSP90AA1 can lead to oncoprotein degradation and disruption of oncogenic pathways.[33,34] HSP90AA1 typically carries out its role by engaging with other proteins. However, it is not yet clear whether circRNAs can bind to HSP90AA1, modulate its biological function, and thereby influence the development of BC.

Our team has mainly conducted research on new biomarkers for breast cancer. In this study, our experimental data displayed that hsa_circ_0000520 promoted the growth, proliferative capacity, and invasive migration of BC cells. Further mechanistic analyses revealed that hsa_circ_0000520, as a newly identified interacting partner of HSP90AA1, cooperated with HSP90AA1 to induce cell metastasis, ultimately leading to enhance tumor metastasis. Furthermore, through conducting functional rescue experiments using the HSP90AA1 inhibitor Tanespimycin, we revealed the dual mechanisms by which hsa_circ_000052 functions in breast cancer: its promotion of cell proliferation is independent of the activity of HSP90AA1, while its key function of driving cell migration and invasion is closely related to HSP90AA1. This indicates that there is a functional synergy between the hsa_circ_000052 and HSP90AA1 in promoting BC metastasis. This is an innovative discovery. Notably, we found that the level of hsa_circ_0000520 was increased in both BC cells, tumor tissues and clinical serum samples and was linked to a more advanced tumor stage and a worse prognosis, which suggests that it could act as a valuable biomarker for diagnosing BC.

Methods and Materials

Clinical Samples

This study was approved by the Ethics Committee of Shaoxing People's Hospital and was conducted in accordance with the Declaration of Helsinki. Before samples were collected, patients had signed the appropriate written consent forms. Forty-two pairs of BC and nearby fresh tissues were collected and frozen right away stored at - 80 °C. Serum samples were obtained from 100 healthy participants, 100 individuals with benign breast disease, and 128 breast cancer patients, and RNA was extracted within two hours. Clinicopathological and molecular features were documented and then analyzed.

Cell Lines

Human normal breast epithelial MCF-10A and breast cancer MCF-7, MDA-MB-231, and HS578T were acquired from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). MCF-10A were cultivated in MEBM BulletKit medium (Lonza, Basel, Switzerland), MCF-7, MDA-MB-231, HS578T were cultured in DMEM with high glucose medium (Gibco, USA) with 10% FBS (ExCell, USA) and 1% penicillin and streptomycin (Biosharp, China). Tanespimycin (MCE, China), an effective HSP90AA1 inhibitor, was used to treat cells at a concentration 1.79 μM for 48 hours, which effectively inhibited the protein HSP90AA1. All cells were placed in 5% CO_2 incubator at 37 °C. The used cells were authenticated for the described experiments (including the % match result and method used) and have not been previously reported as misidentified or mycoplasma contamination.

Cell Lentiviral Transfection

Lentiviruses for overexpression and knockdown of hsa_circ_0000520, as well as negative control, were procured from Genechem, Shanghai, China. Firstly, the MDA-MB-231 were enzymatically digested and inoculated into 6-well plates. The culture was continued to ensure that the seeding density reached 20% for infection. Then, following the protocol provided by the manufacturer for the slow virus infection of cells (Genechem, Shanghai, China), stable cell lines were prepared. Finally, the stable cell lines constructed above were selected using puromycin to obtain the desired target cell lines.

Genomic DNA Isolation

Cells were cultured and when they reached 80% to 90% confluence, genomic DNA was extracted following the QIAamp DNA Mini Kit (Qiagen, Germany) instructions.

Quantitative Reverse Transcription PCR (RT-qPCR)

RNA was extracted from cell lines, BC tissues, and blood samples using Trizol (Invitrogen, USA) following the manufacturer's guidelines. A NanoDrop2000 (Thermo Fisher Scientific) was utilized to determine RNA quantity and purity. The cDNA was prepared and qPCR analyses were conducted with TB Green qPCR Mastermix (TaKaRa, Japan) on an ABI 7500 real-time PCR Platform (Thermo Fisher Scientific). Quantification of hsa_circ_0000520 expression in cells, tissues, and serum samples using standard curves established with absolute plasmid DNA standards. Detailed sequences of primers can be found in Table S1.

Nucleocytoplasmic Separation Experiment

Nuclear and cytoplasmic extracts were derived from breast cancer cells using the PARIS™ Kit (Thermo Fisher Scientific, USA). Later, RT-qPCR was performed to quantify the expression of hsa_circ_0000520, RPPH1 along with the internal reference molecules GAPDH and U6. Detailed sequences of primers can be found in Table S1.

RNase R Experiment

Cells were proliferated until approximately 90% confluency was achieved. Following this, according to the manufacturer's instructions from the GSPure®RNase R kit (Geneseed, Guangzhou, China), one microgram of total RNA with or without 0.5 μL of RNase R were incubated at 37 °C for 15 min and subsequently 70 °C for 10 min. The resulting RNA was subsequently purified and then analyzed by RT-qPCR.

Actinomycin D Experiment

The cells continued to grow until confluence reached 70% before treatment. Subsequently, the cells were treated with 10 μg/mL actinomycin D (Sigma, USA) and harvested at the designated time intervals. RNA stability was evaluated by RT-qPCR and normalized to the values measured in the mock-treated cells at the 0-hour time point.

Cell Proliferation Assay

Cells were placed at 3000 cells per well and cultured 20 h to 26 h. The proliferation index was measured using CCK-8 kit (Abbikine, China) at 450 nm every 24 h.

Colony Formation Assay

Cells were seeded at 1000 cells per well and incubated for 7 days. After treatment with 4% paraformaldehyde (SCRC, China) for 30 min, cell colonies were stained by crystal violet (Servicebio, China) for 20 min and colonies in each group were photographed and subsequently enumerated.

Wound Healing Assay

Cells were cultured until they reached 95% to 100% confluence and scratched in the middle of the wells, then cultured in 1% serum medium. After 24 h of incubation, wound widths were quantified at three independent sites for each group and normalized to the control group.

Transwell Assays

For Transwell migration assay, cells at 5×10^4 /well in serum-free medium were added into upper chamber and the complete medium with 10% FBS was added into the lower chamber using Transwell 24-well chambers (Corning, with 8μm pore diameter). The transwell-containing plate was incubated for 24 h and fixed the lower surface of the membrane with 4% paraformaldehyde (SCRC, China). After the fixation, the transwell chambers were stained with crystal violet (Servicebio). Each well was counted and photographed. For matrigel invasion assay, the chamber was pre-coated with diluted matrigel (Corning, 1:8 dilution, 60 μL/well). The cell inoculation density and experiment were identical to those employed in the Transwell migration assay described above.

Chromatin Isolation by RNA Purification Assay and Mass Spectrometry

The experiment was performed in accordance with the Thermo Magnetic RNA-Protein Pulldown Kit instructions (Thermo Fisher Scientific, USA). We created biotin-tagged 5′ oligonucleotide probes aimed at the hsa_circ_0000520 binding site. The biotinylated hsa_circ_0000520 was captured using streptavidin magnetic beads and incubated with cell lysates overnight at 4 °C. Next, the samples underwent washing and elution procedures. The resulting materials were analyzed by MS and further confirmed using WB. The probe sequences are presented Table S2.

RNA Immunoprecipitation Assay

Experiment was performed according to the guidelines provided by the Millipore Magna RIP RNA Binding Protein Immunoprecipitation Kit (Millipore, USA). The lysates from MDA-MB-231 cells were prepared and incubated overnight at 4 °C with 5 μg of the target protein antibody. Following incubation with proteinase K, subsequently RT-qPCR was analyzed.

Western Blotting

Cells were grown until they were 90% confluent. Cells were lysed with RIPA buffer (Solarbio, China) and protease inhibitor (Roche, CA, USA). Subsequently, the extracted proteins were separated by 12% SDS-PAGE and transferred to a 0.45 μm PVDF membrane (Roche, USA). After blocking with 5% nonfat milk, primary antibodies with antibody dilution buffer (Vazyme, China) were incubated overnight at 4 °C. After washing with 1×TBST for five times, the membrane was incubated with the HRP-conjugated secondary antibody (Invitrogen, USA). The protein levels were detected using a chemiluminescence gel imaging system (GE, USA). β-actin was used as the internal control. All primary antibodies and their corresponding dilutions were listed in Table S3.

Statistical Methods

Data were analyzed statistically using SPSS software 22.0 (IBM, SPSS, USA) and GraphPad Prism 8.0 (GraphPad Software, USA). The experiments were performed three times, and the outcomes are shown as the mean ± SD from three separate tests. Count data were analyzed using the chi-square test or Fisher's exact probability method. Student's *t*-test and χ^2-test were carried out to analyze the differences. The value of $P<0.05$ was regarded as statistically significant.

Results

Hsa_circ_0000520 is a Circular RNA and is Highly Expressed in BC

In order to identify the function of a novel circRNA in BC, bioinformatics analysis was conducted. Drawing on the evaluation of the GSE101123 microarray, which is associated with BC, and applying the standards of | log2 (fold change) | > 2 and $P < 0.05$, we confirmed seven circular RNAs that were downregulated and twelve that were upregulated (Figure 1A), among which hsa_circ_0000520 exhibited markedly elevated expression in malignant breast tissue samples (Figure 1B). According to information from circBase (http://circrna.org/), the circRNA listed as hsa_circ_001846 corresponds to hsa_circ_0000520, which has been documented to enhance initiation and progression of BC. To corroborate these findings, RT-PCR was carried out, which confirmed the circular structure of hsa_circ_0000520. Subsequent Sanger sequencing corroborated its match to the reference annotation (Figure 1C). PCR analysis showed that divergent primers amplified hsa_circ_0000520 in cDNA but failed to generate a product in genomic DNA, supporting its circular RNA structure (Figure 1D). RNase R is an enzyme that targets and breaks down linear RNA molecules, but it does not affect circular RNAs. RNase R exonuclease digestion resistance verified that hsa_circ_0000520 was a loop structure (Figure 1E). Upon actinomycin D treatment, hsa_circ_0000520 displayed markedly higher stability compared with its linear counterpart RPPH1 (Figure 1F). Furthermore, nuclear-cytoplasmic localization assays indicate that this circRNA is primarily localized in the cytoplasm of HS578T cells (Figure 1G). Collectively, these results confirmed that hsa_circ_0000520 is a bona fide circRNA. To further verify the dysregulated expression pattern of hsa_circ_0000520 in BC, RT-qPCR was performed on 42 pairs of breast cancer tissues and matched normal tissues. The results showed that hsa_circ_0000520 was highly expressed in BC tissues (Figure 1H). Moreover, the clinical basic informations of BC patients were summarized in Table 1. We found that increased expression of hsa_circ_0000520 showed a strong association with more advanced TNM classification and the triple-negative breast cancer (TNBC) subtype. Similarly, hsa_circ_0000520 level was elevated in a series of BC cells (Figure 1I). HS578T and MDA-MB-231 cells, which exhibited higher hsa_circ_0000520 level, were selected for the follow-up experiments.

Serum hsa_circ_0000520 Levels Exhibit Increased in BC Patients and are Related to BC Patients Clinicopathological Characteristics

To evaluate hsa_circ_0000520 in greater detail, we utilized RT-qPCR to quantify its expression levels in three groups of serum samples: healthy individuals (n = 100), individuals with benign breast disease (n = 100), and patients diagnosed with breast cancer (n = 128). Following the experimental methodology for performing absolute fluorescence quantitative PCR, using gradient dilution, a standard curve was generated based on RNA concentration and cycle threshold (Ct) values to determine the copy number of hsa_circ_0000520 in serum (Figure 2A). Notably, the level of hsa_circ_0000520 detected in the serum of healthy individuals was the lowest. In contrast, patients with BC exhibited substantially higher serum concentrations of hsa_circ_0000520 than both healthy controls and individuals diagnosed with benign breast disease (Figure 2B). In addition, higher levels of hsa_circ_0000520 expression were found among serum specimens obtained from advanced-stage BC cases (Figure 2C). Next, within the cohort, individuals with breast cancer were divided into two groups- lower and higher serum hsa_circ_0000520 expression- each comprising 64 cases, based on the median circRNA value. Further analysis of the clinical profiles of the two expression groups indicated that higher hsa_circ_0000520 expression was linked to more advanced TNM classification ($P = 0.003$) and molecular subtype ($P = 0.002$) in Table 2.

Hsa_circ_0000520 Facilitates BC Cell Proliferation, Migration, and Invasion

To explore the functional significance of hsa_circ_0000520 in BC, we generated a lentiviral overexpression vector and a junction-specific shRNA lentivirus to upregulate or knock down hsa_circ_0000520 respectively. Post-transfection, green fluorescent protein expression was observed in more than 90% of the cells. (Figure S1). RT-qPCR results validated efficient overexpression or suppression of hsa_circ_0000520 in BC cells (Figure 3A and B). Growth curves analysis performed using CCK-8 assays indicated that increasing hsa_circ_0000520 levels markedly boosted the proliferative capacity of MDA-MB-231 cells, whereas decreasing it inhibited cell growth (Figure 3C and D). Consistent with the CCK-8 results, colony formation assays further elucidated that the proliferative capabilities of BC cells were significantly

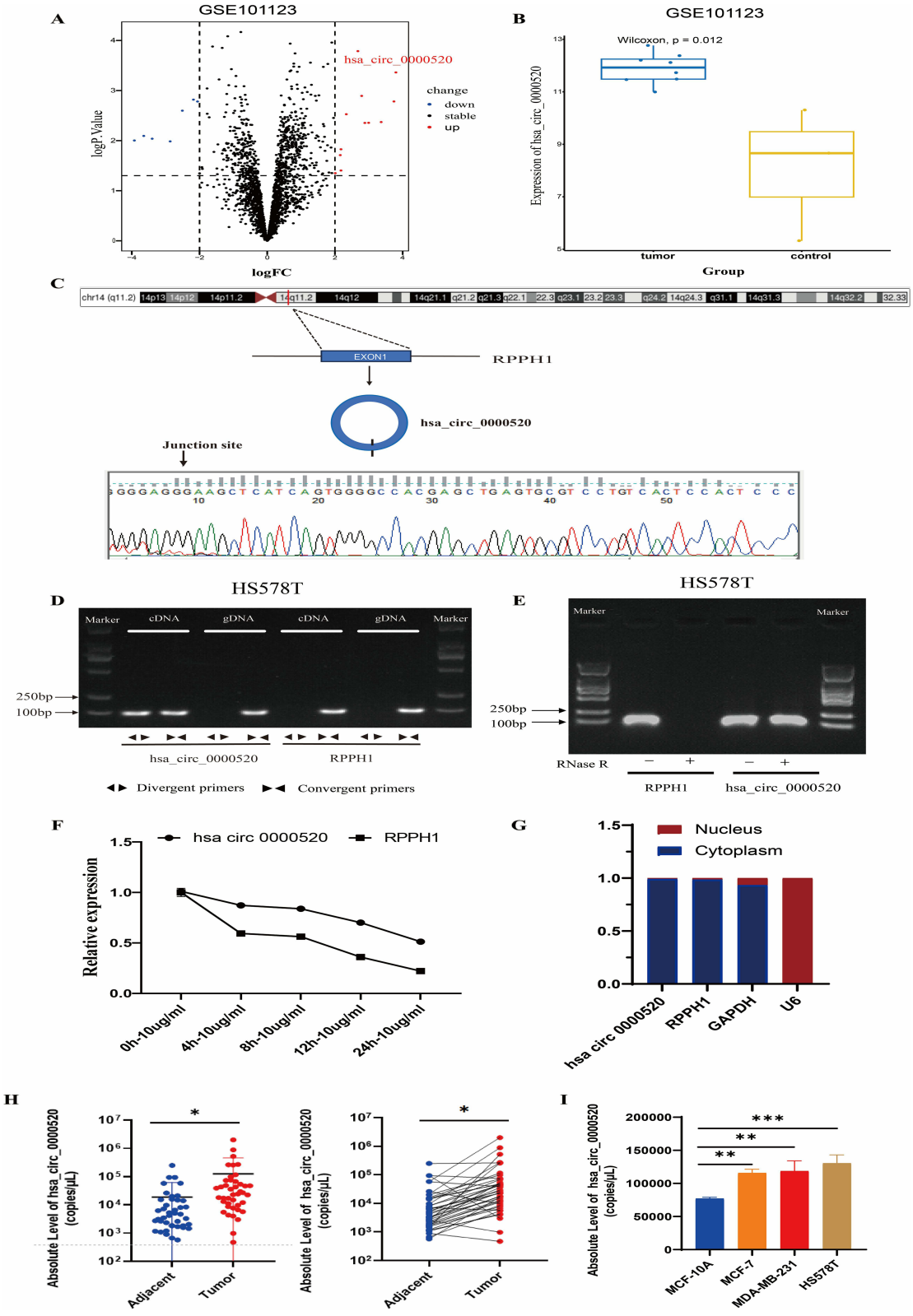

Figure 1 Hsa_circ_0000520 is verified and characterized in BC cells. (**A**) Analysis of the GSE101123 microarray through bioinformatic approaches revealed a set of expressed circRNAs, among which hsa_circRNA_0000520 was detected. (**B**) Expression levels of hsa_circ_0000520 in BC specimens were analyzed using data from GSE101123. (**C**) Sanger sequencing confirmed the sequence of this circRNA. (**D**) Detect the presence of hsa_circRNA_0000520 from cDNA and gDNA in HS578T cells by RT-PCR. Divergent primers successfully amplified hsa_circRNA_0000520 only from cDNA. (**E**) The expression profiles of hsa_circ_0000520 and its linear counterpart RPPH1 were examined after RNase R treatment. (**F**) RNA levels of hsa_circ_0000520 and RPPH1 were quantified in HS578T cells exposed to actinomycin D at the specified time point. (**G**) Hsa_circ_0000520 was predominantly distributed in the cytoplasm of BC cells, with U6 and GAPDH used as nuclear and cytoplasmic controls, respectively. (**H**) RT-qPCR assay measured hsa_circ_0000520 expression in 42 pairs tumor and neighboring tissues. (**I**) RT-qPCR assay measured hsa_circ_0000520 expression in various BC cell line. **P*<0.05; ***P*<0.01; ****P*<0.001.

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Table 1 Association Between Levels of Hsa_circ_0000520 Expression and Clinicopathological Characteristics in BC Tissues

Parameters	Total	Expression of hsa_circ_0000520		χ^2	P
		Low	High		
Age					
≤58	21	12	9	0.857	0.355
>58	21	9	12		
TNM stage					
I/II	37	21	16	5.676	**0.017***
III/IV	5	0	5		
Tumor size					
≤2	19	10	9	0.096	0.757
>2	23	11	12		
Lymph node metastasis					
Yes	18	6	12	3.5	0.061
No	24	15	9		
ER					
Positive	35	20	15	4.286	**0.038***
Negative	7	1	6		
PR					
Positive	27	13	14	0.104	0.747
Negative	15	8	7		
HER2					
Positive	14	9	5	1.714	0.190
Negative	28	12	16		
TNBC					
Yes	4	0	4	4.421	**0.035***
No	38	21	17		

Notes: *P < 0.05; Bold values indicate statistical significance.

enhanced by upregulation of hsa_circ_0000520 and markedly impaired by downregulation of hsa_circ_0000520 (Figure 3E and F). Taken together, the findings demonstrate that hsa_circ_0000520 enhances proliferation expansion of BC cells. Subsequently, we utilized scratch and Transwell assays to evaluate how hsa_circ_0000520 influences the motility and invasive capacities of BC cells. The results demonstrated that hsa_circ_0000520 overexpression greatly enhanced MDA-MB-231 cells migration, while its knockdown significantly diminished migration in scratch assays (Figure 3G and H). The abilities of MDA-MB-231 cells to migrate and invade were greatly enhanced by the overexpression of hsa_circ_0000520 and markedly suppressed by its knockdown, as demonstrated in the Transwell migration assays (Figure 3I and J) and invasion assays (Figure 3K and L), respectively. These data indicated that hsa_circ_0000520 enhanced the proliferative, migratory, and invasive behaviors of BC cells.

Hsa_circ_0000520 Directly Interacts with HSP90AA1

To explore how hsa_circ_0000520 mechanistically contributes to breast cancer biology, ChIRP assay was conducted using biotin-tagged hsa_circ_0000520 probes to identify proteins potentially capable of binding to this circRNA.

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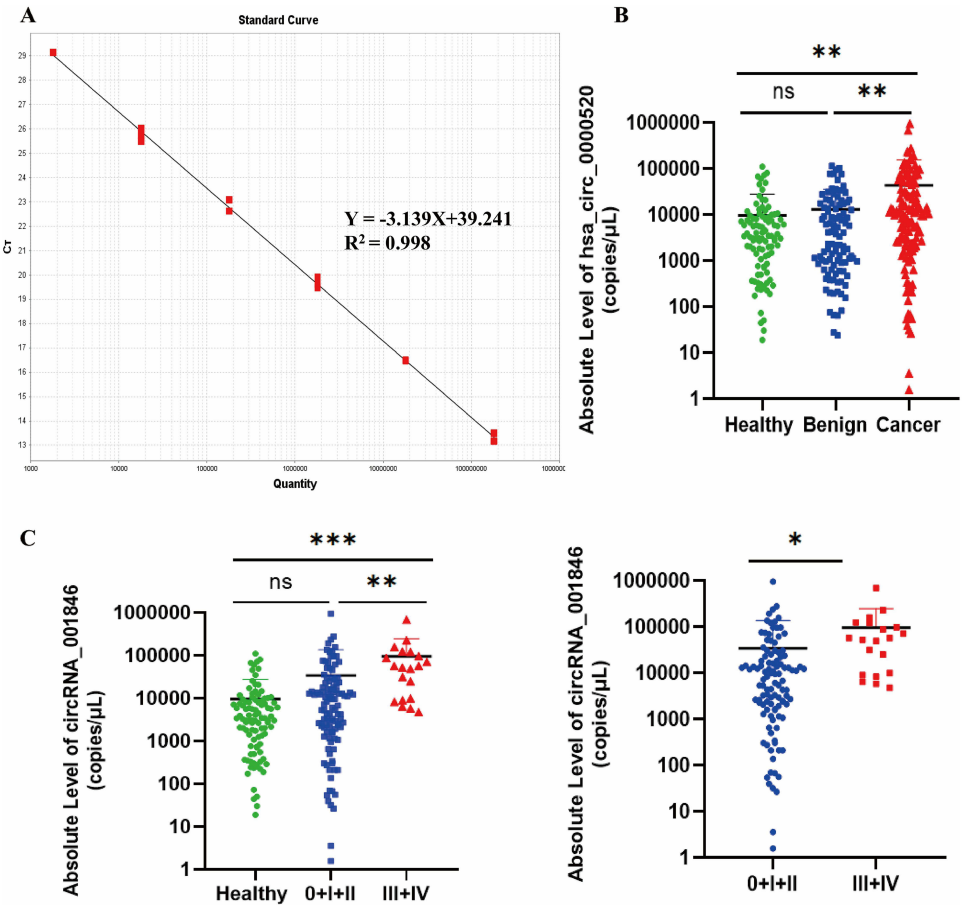

Figure 2 BC patients show increased serum expression of hsa_circ_0000520. (**A**) The standard curve was employed for the purpose of absolute quantitation. (**B**) The serum expression level of hsa_circ_0000520 from healthy people (n = 100), individuals with benign breast disease (n=100), and BC (n = 128) were quantified using RT-qPCR. (**C**) The abundance of hsa_circ_0000520 in different stages of the disease. *P<0.05; **P<0.01; ***P<0.001.
Abbreviation: ns, no significance.

Firstly, ChIRP assay was conducted in MDA-MB-231 cells using biotin-tagged probes (Figure 4A). Then, the mass spectrometry analysis identified 107 proteins that exhibited an association with hsa_circ_0000520 (Table S4). Among the proteins, HSP90AA1 was highlighted as a potential hsa_circ_0000520-interacting protein in BC cells (Figure 4B). The interaction was further validated through WB, which confirmed the presence of HSP90AA1 in the protein complex pulled down by hsa_circ_0000520 (Figure 4C). RNA immunoprecipitation (RIP) experiments further validated this interaction (Figure 4D).

Hsa_circ_0000520 Cooperates with HSP90AA1 to Promote Breast Cancer Cells Invasion and Metastasis

Previous studies have confirmed that HSP90AA1 effectively facilitates the invasion and metastasis of BC. Nevertheless, it is still unknown how it functions together with the bound cicrRNAs. To further explore the influence of HSP90AA1 on the biological functions of hsa_circ_0000520, in vitro rescue experiments were conducted by modulating HSP90AA1 expression in cells. Cells were treated with tanespimycin, an HSP90AA1 inhibitor, for 48h to inhibit the HSP90AA1, the effects of hsa_circ_0000520 on BC cells proliferation and invasion were subsequently re-verified (Figure 5A). CCK-8 indicated that after inhibition of HSP90AA1, the upregulation of hsa_circ_0000520 still significantly enhance MDA-MB-231 proliferation, and similarly, its knockdown could consistently inhibit cell growth (Figure 5B and C). The colony formation assay results also reached the same conclusion. When HSP90AA1 was inhibited, the proliferative ability of BC cells was also still significantly

Table 2 The Relationships Between the Expression of Serum Hsa_circ_0000520 and Clinical Characteristics in BC Patients

Parameters	Total	Expression of hsa_circ_0000520		χ^2	P
		Low	High		
Age at diagnosis					
≤55	67	38	29	2.537	0.111
>55	61	26	35		
TNM stage					
0/I/II	105	59	46	8.957	**0.003***
III/IV	23	5	18		
Tumor size					
≤2	76	43	33	3.239	0.072
>2	52	21	31		
Lymph node metastasis					
Yes	44	17	27	3.463	0.063
No	84	47	37		
ER					
Positive	93	52	42	4.005	**0.045***
Negative	35	12	22		
PR					
Positive	85	48	37	4.237	**0.040***
Negative	43	16	27		
HER2					
Positive	37	21	16	1.130	0.288
Negative	55	25	30		
unknown	36	18	18		
TNBC					
Yes	12	1	11	9.195	**0.002***
No	116	63	53		

Notes: *$P < 0.05$; Bold values indicate statistical significance.

increased due to the upregulation of hsa_circ_0000520 and was still significantly impaired due to the downregulation of hsa_circ_0000520 (Figure 5D and E). These experimental results indicate that inhibiting HSP90AA1 has no effect on the breast cancer cells proliferation when hsa_circ_0000520 is present. However, after inhibiting HSP90AA1, different phenomena were observed in the scratch test and Transwell assays used to detect the effect of hsa_circ_0000520 on the migration and invasion of BC cells. After inhibiting HSP90AA1, neither overexpression nor knockdown of hsa_circ_0000520 affected the migration of BC cells in the scratch assays (Figure 5F and G). Similarly, in the Transwell assays, no significant effects on migration or invasion were observed following either overexpression or knockdown of hsa_circ_0000520 in BC cells (Figure 5H–K). This result indicated that hsa_circ_0000520 facilitates BC cells invasion and metastasis by cooperating with and modulating the function of HSP90AA1, and it is a key oncogenic factor driving the malignant progression of BC.

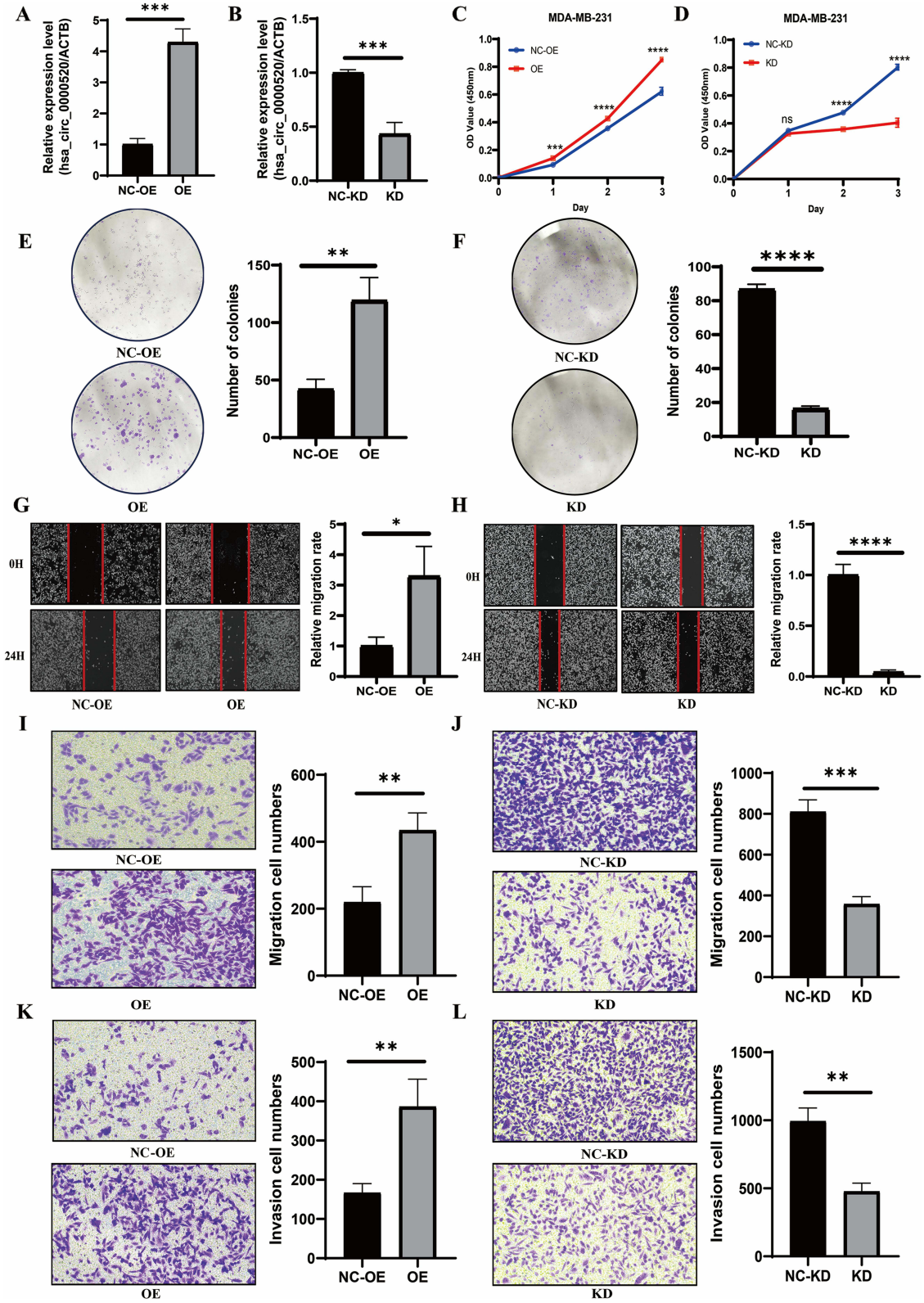

Figure 3 Hsa_circ_0000520 increases proliferation, migration and invasion of BC in vitro. (**A**) Transfection efficiency of the overexpressed hsa_circ_0000520 lentivirus in MDA-MB-231 cells was assessed by RT-qPCR. (**B**) Transfection efficiency of hsa_circ_0000520 lentivirus knockdown in MDA-MB-231 cells was analyzed by RT-qPCR. (**C** and **D**) Cell growth in the distinct MDA-MB-231 groups was quantified through CCK-8 assays. (**E** and **F**) The growth of BC cells was measured by colony formation assay. (**G** and **H**) Scratch wound analysis was used to evaluate changes in MDA-MB-231 cell migration following hsa_circ_0000520 overexpression or knockdown. (**I** and **J**) Evaluation of how hsa_circ_0000520 overexpression and knockdown influence the migratory capacity of MDA-MB-231 cells was conducted using a migration assay (200x). (**K** and **L**) Evaluation of how hsa_circ_0000520 overexpression and knockdown influence the invasive behavior of MDA-MB-231 cells was assessed using an invasion assay (200x). **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001.
Abbreviation: ns, no significance.

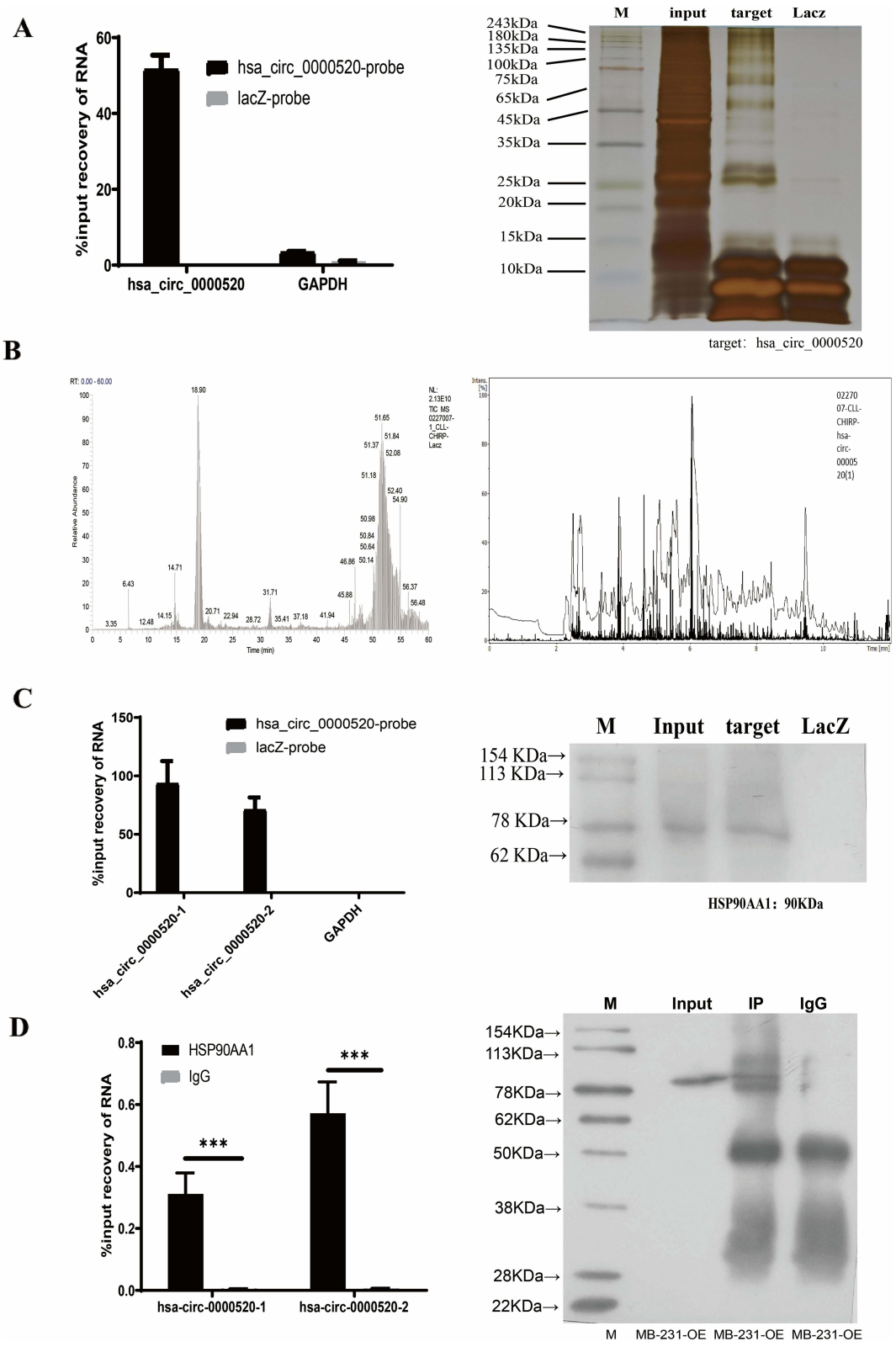

Figure 4 Hsa_circ_0000520 can bind to HSP90AA1. (**A** and **B**) ChIRP assay was conducted with the biotin-tagged hsa_circ_0000520 probe in MDA-MB-231 cell lysates, with subsequent silver staining, and the protein bands were further examined using MS. (**C**) WB analysis of the RNA pulldown material confirmed that HSP90AA1 was captured by hsa_circ_0000520. (**D**) The association between HSP90AA1 and hsa_circ_0000520 is further demonstrated by RIP reverse analysis. ***P<0.001.

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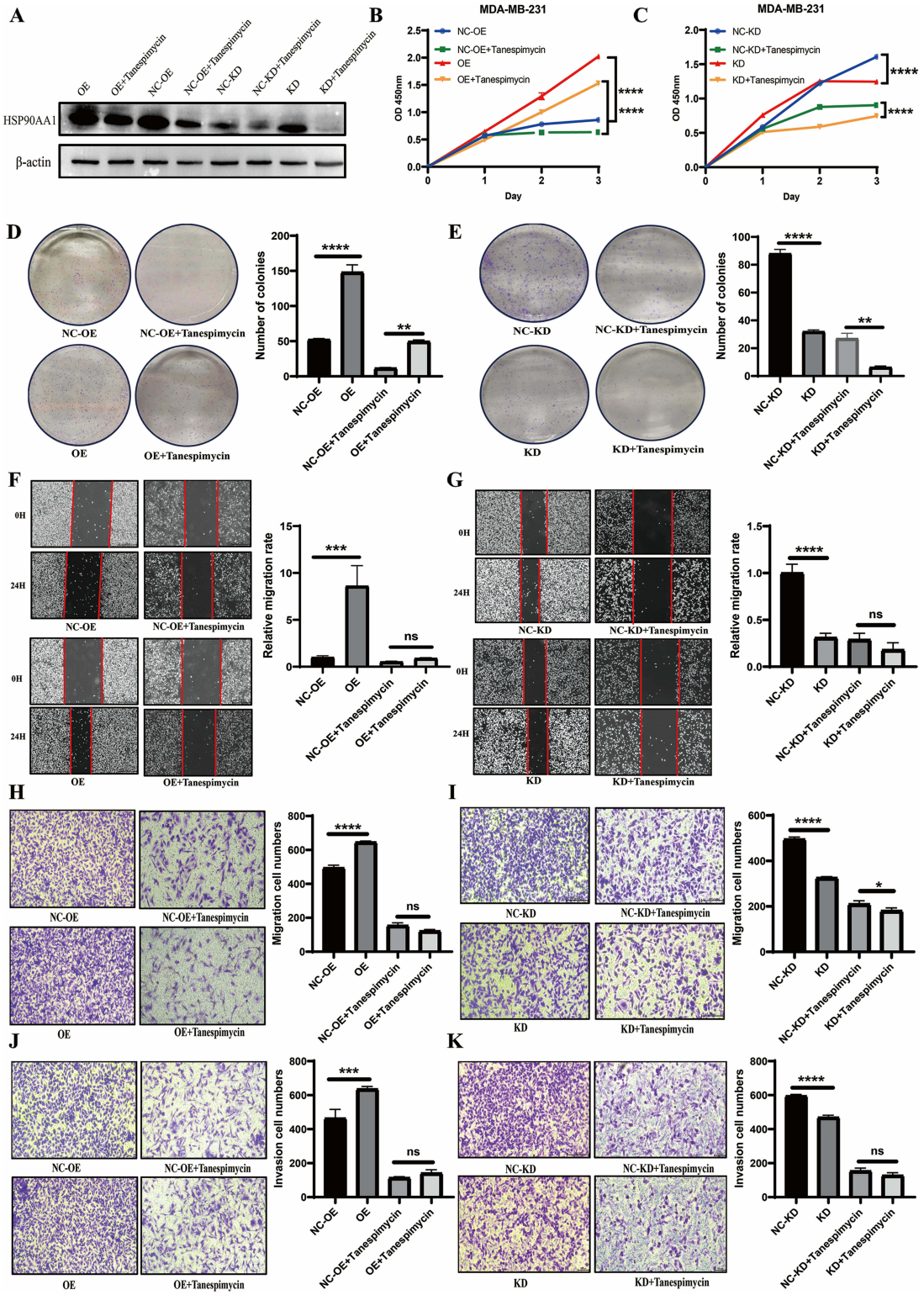

Figure 5 Tanespimycin inhibited the invasion and migration of BC cells. (**A**) After treatment with the HSP90AA1 inhibitor Tanespimycin, the WB experiment verified that HSP90AA1 expression was decreased in BC cells. (**B** and **C**) The CCK-8 assays were employed to determine the viability of MDA-MB-231 cells with hsa_circ_0000520 overexpression or knockdown, treated with or without 1.79 μM Tanespimycin for 48 hours. (**D** and **E**) Colony formation assays were performed to assess MDA-MB-231 proliferation with hsa_circ_0000520 overexpression or knockdown, treated with or without 1.79 μM Tanespimycin for 48 hours. (**F** and **G**) scratch assays were performed on BC cells with hsa_circ_0000520 overexpressed or knocked down, treated with or without 1.79 μM Tanespimycin for 48 hours. (**H** and **I**) The effect of hsa_circ_0000520 overexpression or knockdown, with or without 1.79 μM Tanespimycin treatment for 48 hours, on MDA-MB-231 cell migration was assessed by the migration assays (200x). (**J** and **K**) The effect of hsa_circ_0000520 overexpression or knockdown, treated with or without 1.79 μM Tanespimycin for 48 hours, on MDA-MB-231 cell invasion was assessed using invasion assays (200x). β-actin served as an internal reference. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001.

Abbreviation: ns, no significance.

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Discussion

Breast cancer presents as a multifaceted and diverse disease, with elevated incidence and mortality rates among women around the world. Even though certain breast cancer patients have positive outcomes, tumor progression in others remains a public health concern.[35] Evidence is mounting that circRNAs are key players in the initiation and advancement of diverse malignancies.[6] Nevertheless, the molecular mechanisms through which circRNAs contribute to BC development remain insufficiently clarified.

In this study, we demonstrated that hsa_circ_0000520 was markedly upregulated in BC cell lines, patient tissues, and serum samples through bioinformatics analysis and RT-qPCR experiments. Functionally, the results of our study confirmed that heightened hsa_circ_0000520 expression drives the in vitro expansion and invasiveness of BC cells, while decreased expression exerts an inhibitory effect on these processes. Previous research has indicated that circRNAs engage in direct interactions with various proteins in the cell, enabling them to influence numerous physiological pathways,[36,37] but the research on circRNAs and heat shock proteins in BC is relatively scarce. Through this research, we discovered that hsa_circ_0000520 can interact with the HSP90AA1 through ChIRP assay combined with mass spectrometry for screening, WB and RIP experiments for verification.

Existing reports have established a strong association between HSP90AA1 function and the regulation of cell migration and tumor metastasis.[29] Therefore, in this study, by using the HSP90AA1 inhibitor Tanespimycin to inhibit the HSP90AA1, the impacts of hsa_circ_0000520 on BC cell growth and invasion were analyzed in a comparative manner. The results showed that after inhibiting HSP90AA1, overexpression of hsa_circ_0000520 was still capable of significantly stimulating BC cell proliferation, but failed to promote their invasion and metastasis. This indicates that hsa_circ_0000520 promotes the invasive and metastatic behavior of BC cells mainly by cooperating with HSP90AA1. The experiments that knocked down hsa_circ_0000520 also confirmed this conclusion.

In recent years, circRNAs, due to its high conservation, tissue specificity,[38] temporal specificity[39] and high stability,[40] have gained growing recognition as promising tools for early cancer diagnosis and outcome prediction, have attracted substantial research interest.[9] CircRNAs can exhibit sustained stability within the plasma and serum of patients and are resistant to changes in pH value or freeze-thaw cycles.[6] It is an ideal potential biomarker and therapeutic target for blood-based testing.[41] Here, we explored the potential diagnostic efficacy of hsa_circ_0000520 in breast cancer. We found that the upregulation of this circRNA in serum and tumor samples correlated with tumor differentiation and TNM staging in BC patients. This indicates that hsa_circ_0000520, functioning as a modulator of BC onset or advancement, may possess meaningful functional significance. Considering the small number of enrolled patients, it may cause bias. Therefore, expanding the number of clinical samples is essential for a more indepth assessment of the link between hsa_circ_0000520 and relevant clinicopathological parameters.

Conclusively, we identified hsa_circ_0000520, a circular RNA generated from RPPH1. Its increased expression is closely linked to adverse prognostic outcomes in BC. In addition, we confirmed that increasing hsa_circ_0000520 expression significantly stimulates BC cells growth, invasiveness, and metastatic behavior. Mechanistically, we have for the first time discovered that hsa_circ_0000520 binds to HSP90AA1, working together to promote the metastasis and development of BC. Our findings not only highlighted the critical role of hsa_circ_0000520 in the malignant development of BC but also provided a new potential diagnostic biomarker for BC.

Although this study presents novel findings, certain limitations should be acknowledged. First, most functional assays rely heavily on MDA-MB-231 cells, which only represent an aggressive triple-negative context. Further validation in additional cell lines representing other molecular subtypes would be valuable to confirm the broader relevance of our findings. Second, while our transwell and scratch assays provide clear evidence of altered migration, the migration phenotype remain inferred from population-level assays.[42] Additional validation is also necessary to comprehensively evaluate the contribution of hsa_circ_0000520 to tumor progression. Third, the sample size was relatively small. Considering that the menopausal status, germline mutation background and subtype distribution could alter RNA abundance patterns in serum,[43,44] future studies will need to be conducted using a larger sample group with well-annotated data to validate the biomarker potential of hsa_circ_0000520 across different patient subgroups. Fourth, the use of tanespimycin suppress HSP90 activity and abolish the migration phenotype, which support cooperation between the circRNA and HSP90AA1. Still, pharmacologic inhibition alone leaves open the possibility of broader stress-response

effects. A more convincing validation involving genetic perturbations should be included in subsequent experiments, such as siRNA or CRISPR-based knockdown of HSP90AA1 followed by circRNA overexpression rescue. Finally, the precise binding sites and downstream signaling of the hsa_circ_0000520–HSP90AA1 interaction remain unclear. A useful follow-up experiment needs to be carried out to determine which region mediate the interaction and to clarify the promising therapeutical targets for breast cancer in the future.

In the context of modern precision oncology, metastasis biomarkers are increasingly being integrated into multi-omics frameworks to improve prediction and patient management.[45] How to position hsa_circ_0000520 within this broader field of biomarkers and realize its potential for clinical application could become the focus of our subsequent research.

Conclusion

In summary, our results advance insight into circRNA–protein interactions and support the clinical relevance of hsa_circ_0000520 as a biomarker for liquid biopsy and a potential target for metastatic breast cancer management. Future research should focus on validating these findings in larger clinical cohorts and in vivo models, and on elucidating the precise molecular details of the hsa_circ_0000520-HSP90AA1 complex to facilitate targeted therapeutic strategies.

Abbreviations

BC, breast cancer; CircRNAs, circular RNAs; HSPs, heat shock proteins; HSP90AA1, heat shock protein 90 alpha; ChIRP, chromatin isolation by RNA purification assay; MS, mass spectrometry; WB, Western blotting; ACTD, actinomycind experiment; gDNA, genomic DNA; RT-qPCR, quantitative reverse transcription PCR; RIP, RNA immunoprecipitation assay; Ct, cycle threshold; ns, no significance.

Data Sharing Statement

All of the data and materials in this paper can be obtained from the corresponding author when requested.

Ethics Approval and Consent to Participate

The study was approved and supervised by the Ethics Committee of Shaoxing People's Hospital (approval no. 202307101, date: 09.01.2023) based on the Helsinki Declaration. Written informed consent was obtained from all participants.

Patient Consent for Publication

Consent for publication was obtained from all participants.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

All the authors state that there are no conflicts of interest in this work.

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