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ORIGINAL RESEARCH

Genetic Correlation of miR-423 Polymorphism rs8067576 with Progression and Prognosis of Triple-Negative Breast Cancer

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Background: Single nucleotide polymorphisms (SNPs) of microRNAs can affect the functional activity of microRNA, thereby relating to disease susceptibility.

Objective: The study systematically examined the impact of miR-423 rs806757 SNP on triple-negative breast cancer (TNBC) risk and severity and dissected the attendant molecular mechanism.

Materials and Methods: Three hundred TNBC patients and 300 controls were genotyped for miR-423 rs806757, and its association with relapse-free survival (RFS) and 5-year survival was analyzed. CCK-8/Transwell assays quantified the variant's influence on tumor cell proliferation, migration and invasion. In-silico target prediction followed by GO/KEGG profiling mapped the downstream pathways.

Results: A significant difference was detected in the genotype distribution of rs8067576 polymorphism between TNBC and controls. And cases harboring rs8067576 AA allele exhibited a higher prevalence of tumors >5 cm, lymph-node involvement, and higher stage (III–IV). AA genotype carriers displayed markedly reduced RFS and 5-year overall survival, and held a conspicuous rise in miR-423-5p levels compared with patients bearing alternative genotypes. Cell-based assays revealed that introducing rs8067576-A allele into tumor cells robustly boosted tumor-cell proliferation, motility, and invasiveness relative to T allele. Subsequent target prediction and pathway enrichment identified Wnt and Ras signaling as the principal downstream effector modules of miR-423-5p.

Conclusion: MiR-423 rs8067576 was a susceptibility locus for TNBC and linked to earlier relapse and shorter 5-year survival. Rs8067576 boosted miR-423-5p expression, thereby enhancing tumor-cell proliferation, motility, and invasiveness.

Keywords: miR-423-5p, single nucleotide polymorphism, triple-negative breast cancer, genetic susceptibility, cellular function

Introduction

Triple-negative breast cancer (TNBC) stands out as a strikingly heterogeneous subtype of breast cancer.[1] Its core characteristic is that the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2) all show negative expression.[2] Consequently, patients are deprived of the therapeutic benefits of both endocrine manipulation and HER-2-targeted drugs.[3] With no targeted options, TNBC faces a flare of relapses during the first five years.[4] TNBC accounts for approximately 15%–20% of all breast cancer cases, with a 5-year survival rate of only around 60%,[5] which seriously threatens the health of women.

In recent years, with the development of precision medicine, the role of genetic factors in the pathogenesis, progression and prognosis of TNBC has gradually become a research hotspot. microRNA (miRNA), as a type of non-coding RNA with a length of approximately 22 nucleotide non-coding strands, silences genes by docking onto the 3'-untranslated region (3'-UTR) of target genes. It then participates in key biological processes such as cell proliferation,

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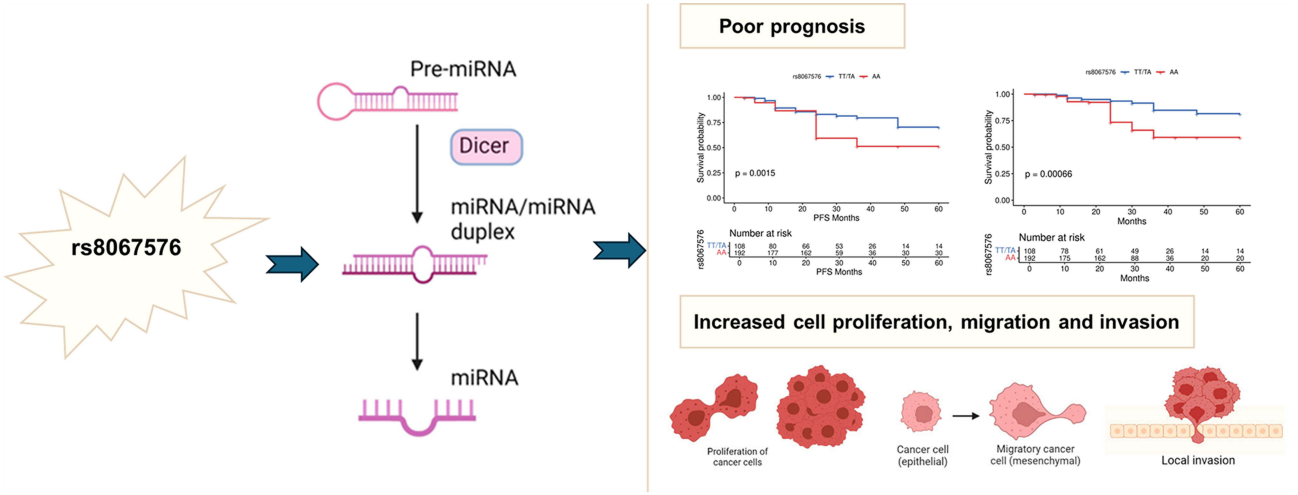
Graphical Abstract



apoptosis, invasion and metastasis.[6] Existing studies have confirmed that single-nucleotide polymorphisms (SNPs) of miRNAs can affect the functional activity of miRNA by altering its transcriptional efficiency, processing and maturation process, or the binding ability to target genes.[7,8] Ultimately, it is closely related to the genetic susceptibility of tumors, disease progression and prognosis.

MiR-423 is a type of miRNA that is abnormally expressed in various malignant tumors. Recent studies have confirmed that it plays a significant role in the occurrence, development, and treatment resistance of TNBC. Research indicates that miR-423-5p upregulates MARCH2 by competitively binding to lncRNA linc00707, activates the PI3K/AKT/mTOR pathway, promotes the proliferation, migration, and invasion of TNBC cells, and can also affect the progression of TNBC by regulating autophagy.[9] Another study shows that miR-423 is a key regulatory factor for chemotherapy resistance in TNBC, and the core mechanism is that miR-423-5p activates proliferation, metastasis, and anti-apoptosis-related signaling pathways.[10] These studies confirm the regulatory role of 423 in the pathogenesis of TNBC and elucidate its regulatory mechanisms. At the clinical level, the expression level of miR-423-5p in the plasma of TNBC patients is associated with pathological complete response (pCR), suggesting that it can be used to predict the response of patients to neoadjuvant chemotherapy (NACT).[11] Moreover, high expression of miR-423-5p is related to the invasiveness and metastatic ability of TNBC, further supporting its potential as a biomarker. Another study shows that the cytosine-to-adenine (C > A) mutation of the SNP rs6505162 of miR-423 can enhance the proliferation, migration, invasion ability, and chemotherapy resistance of breast cancer cells by upregulating the expression of mature miR-423, thus leading to a significant elevation in the risk of TNBC.[12] MiR-423 rs8067576 is an SNP site located in the coding region of the miR-423 gene. Another study found that the rare T allele of the rs8067576 polymorphism can cause structural changes in the pre-miR-423 sequence, thereby affecting the maturation process of miR-423.[12] Functionally, the T allele is more capable of promoting cell proliferation than the A allele, while the A allele has a more significant inhibitory effect on the translation of the proliferation-related protein Pa2g4 in recurrent spontaneous abortion,[12] suggesting that the rs8067576 polymorphism may be involved in the regulation of TNBC by affecting the function of miR-423.

This study aimed to use miR-423 rs806757 as a genetic anchor point to systematically analyze the quantitative association between its genotypes and the tumor size, lymph node invasion (LNM), and clinical stage of TNBC, and to quantitatively evaluate its prognostic weight for disease-free survival and overall survival (OS). The goal was to establish this polymorphism locus as an independent genetic predictor of TNBC progression and outcome and to provide a genetic

entry point for further elucidating the molecular mechanism of miR-423 regulatory network in the malignant progression of TNBC.

Materials and Methods

Study Subjects

The sample size was calculated using the OpenEpi tool, with parameters set as follows: odds ratio (OR) = 1.5, significance level (α) = 0.05, and power (1-β) = 0.8. After accounting for a 10% attrition rate, the calculation revealed a minimum requirement of 200 cases and 200 controls. The study population comprised TNBC patients admitted to the Traditional Chinese Medical Hospital of Wenling affiliated to Zhejiang Chinese Medical University between January 2019 and June 2021. All patients had peripheral venous blood samples collected after diagnosis but before surgery or initial anticancer therapy. Complete clinical and serological records were available for all patients. To ensure the homogeneity and reliability of study populations, strict inclusion and exclusion criteria were applied to both TNBC cases and healthy controls. Patients were included in the TNBC case cohort if they 1) had pathologically confirmed triple-negative breast cancer (ER-, PR- and HER2-negative), 2) were aged 18 years or over, 3) provided informed consent, 4) have not received neoadjuvant chemotherapy or radiotherapy at the time of initial diagnosis and 5) to provide a suitable peripheral blood sample. Exclusions applied to individuals with other malignancies, severe comorbidities, prior treatments within the preceding 3 months that could interfere with genetic testing, pregnancy or lactation, and those with unsuitable blood samples. Healthy control group inclusion criteria: 1) No history of malignant tumors or severe chronic diseases; 2) Age matched with the case group; 3) Female gender and consistent racial background as much as possible; 4) Individuals able to provide qualified peripheral blood samples and sign informed consent forms. Exclusion Criteria: 1) History of familial tumors or benign breast lesions; 2) Infection or inflammatory disease within the past 3 months; 3) Pregnancy/lactation or hormone replacement therapy; 4) Individuals with unsuitable blood samples. Ultimately, 300 healthy controls (HC) and 300 histologically TNBC patients were enrolled in this study. Demographic and clinicopathologic variables were retrieved from electronic medical records and summarized in Table 1. The study protocol was approved by the Traditional Chinese Medical Hospital of Wenling affiliated to Zhejiang Chinese Medical University Ethics Committee, and written informed consent was obtained from every participant.

Table 1 The Basic Characteristics of the Study Groups

Parameters	HCs (n = 300)	TNBC (n = 300)	*P* Value
Age, year			
>50	184	192	
≤50	115	108	
Menopause			
No	167	183	
Yes	132	117	
Histological subtype	/		
Ductal		254	
Lobular		46	
Tumor size, cm	/		
≤5		211	
>5		89	
LNM	/		
Negative		177	
Positive		123	
TNM	/		
I–II		183	
III–IV		117	

Abbreviations: TNBC, triple-negative breast cancer; HC, healthy control; TNM, tumor-node-metastasis; LNM, lymph node metastasis.

Follow-Up Assay

The entire TNBC group completed a five-year follow-up. Overall survival (OS) was calculated from treatment initiation to death from any cause. Relapse-free survival (RFS) was defined as the interval from treatment start to first recurrence (local, regional, or distant) or death.

DNA Extraction and Genotyping Method

Five milliliters of venous blood were collected from each subject and stored in EDTA tubes. Genomic DNA was extracted from whole blood samples using the MolPure® Blood DNA Rapid Extraction Kit (Yeasen, Shanghai) according to the manufacturer's protocol. Genotyping for the rs8067576 polymorphism was performed with the TaqMan™ SNP Genotyping Kit (Applied Biosystems, USA). The total reaction volume was 20 μL, containing 10 μL of 2 × TaqMan Universal PCR Master Mix, 1 μL of 20 × TaqMan SNP Genotyping Assay (primer-probe mix), 1 μL of DNA sample (10 ng/μL), and 8 μL of deionized water.

Cell Lines and Transfection

The cells used in this study include the human normal breast epithelial cell line MCF10A, as well as two human breast cancer cell lines BT-20 and MDA-MB-231. Both cells were purchased from the American Type Culture Collection (ATCC). All cells were routinely cultured in complete medium supplemented with 10% fetal bovine serum (FBS) to provide the nutrients and growth factors necessary for cell growth. The cell culture environment was maintained in a constant temperature and humidity incubator at 37°C with 5% carbon dioxide (CO_2), which could simulate the physiological environment in vivo and ensure the cells were in a stable growth state.

For cell transfection operations, the Lipofectamine 2000 reagent (Invitrogen) was used according to the manufacturer's recommended standard protocol. The expression vectors containing pre-miR-423 rs8067576-T or A allele were designed and provided by Thermo Fisher Scientific Co. (Waltham, MA, USA). Before transfection, the cells were seeded in appropriate culture vessels, and transfection was performed when the cell confluence reached 70%–80% to improve the transfection efficiency.

RT-qPCR

TRIzol extracted total RNA from plasma and cultured cells alike. RNA purity was assessed by the A260/A280 ratio, which ranged from 1.8 to 2.0, indicating high-quality RNA. cDNA was then synthesized with the PrimeScript RT Reagent Kit (Takara Bio USA), and amplification was performed using SYBR Green PCR Master Mix from Applied Biosystems (Foster City, CA, USA). Target-gene abundance was quantified via $2^{-\Delta\Delta Ct}$, normalized to U6.

CCK-8 Assay

Twenty-four hours after transfection, the cells were harvested with the density to 5×10^3 cells/well. Then 100 μL of the cell suspension was seeded into each well of a 96-well plate, preparing five replicate wells for every group. At the indicated time points (0, 24, 48 and 72 h of incubation), 10 μL of CCK-8 solution was added to each well and the absorbance at 450 nm was recorded.

Transwell Assays

Transwell assay was employed to gauge the migratory and invasive capacity of cells. 5×10^3 cells were plated in the upper compartment of a 24-well Transwell for migration assay, while 1×10^4 cells were plated onto Matrigel-coated inserts. The lower chamber contained complete medium with 10% FBS as a chemoattractant. Following 48 h incubation, non-invading residual cells on the upper surface were gently wiped away using a cotton swab. Cells that had traversed the filter were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet. After rinsing and air-dried, cells were enumerated in five randomly selected high-power fields per insert under a light microscope.

Functional and Pathway Annotation of Target Genes of miR-423-5p

First, three mainstream databases (TargetScan, miRDB and microT) were independently queried to predict the downstream targets of the miR-423-5p. To obtain a more reliable candidate gene set, the prediction results from these three databases were intersected, and the overlap was visualized with a Venn diagram. GO and KEGG enrichment analyses were employed to assign functional terms and pathways to the intersecting target genes.

RIP Assay

Log-phase breast cancer cells (BT20 and MDA-MB-231) were subjected to RNA immunoprecipitation (RIP) using an Ago2 antibody. The Ago2-RNA complex was isolated via magnetic bead capture, and the bound RNA was extracted. The enrichment levels of miR-423 and its target genes were then detected by RT-qPCR, with the IgG group serving as a negative control and the relative enrichment fold was calculated to validate their intracellular association.

Luciferase Reporter Assay

Wild-type/mutant (WT/MUT) luciferase reporter plasmids containing the target gene's 3'UTR were co-transfected into 293T cells with miR-423 mimic, inhibitor or their negative controls (mimic-NC or inhibitor NC). After 48 hours of culture, firefly luciferase and sea squirt luciferase activities were measured. Sea squirt luciferase activity served as an internal control for normalization. Changes in relative luciferase activity were used to validate the direct binding of miR-423 to the target gene's 3'UTR.

Statistical Analysis

SPSS 21.0 served as the statistical platform. Between-group differences were evaluated by an independent-samples *t*-test or χ^2-test as appropriate. Survival curves were generated by Kaplan–Meier (KM) analysis. The potential prognostic factors were identified by Cox proportional-hazards regression, and the hazard ratios (HR) and corresponding 95% confidence intervals (CI) were computed to assess the magnitude and statistical significance of prognostic correlations. Significance was set at a two-sided P value <0.05.

Results

Basic Characteristics of the Study Groups

Table 1 presents a comparison of the basic characteristics of the two enrolled study groups. Two basic characteristics including age and menopausal status showed no significant differences between the two groups ($P > 0.05$). In addition, the histopathological features of enrolled patients were recorded, among 300 TNBC patients, a larger proportion of cases owned small tumor size, negative LNM and low TNM stage.

Comparison of Genotype and Allele Distributions of the miR-423 rs8067576 Polymorphism in TNBC and HC Groups

Table 2 delineates the comparison results of genotype and allele distributions of the miR-423 rs8067576 variant across the two cohorts, the genotype and locus frequency distribution of the control group conformed to the Hardy-Weinberg equilibrium, indicating good reliability of the results. It was ascertained that in comparison with the control group, AA genotype carriers (64.0% vs 55.33%) accounted for a larger share of expenses in TNBC group, while TT carriers (5.00% vs 9.33%) showed a comparatively lower proportion ($P = 0.020$). Given the remarkably over-representation of AA genotype carriers detected in the case group, this genotype was identified to be a potential susceptibility factor (OR = 2.159, 95% CI = 1.115–4.180). Mutually, the A allele was concurrently enriched to a significant extent in TNBC cohort ($P = 0.008$), pointing toward it being a risk-conferring allele (OR = 1.434, 95% CI = 1.097–1.875). Further analysis using genetic models revealed that the genetic association between rs8067576 locus and TNBC was statistically significant under both the dominant and recessive models (all $P < 0.05$), reinforcing the genetic association of this variant to disease susceptibility.

Table 2 The Genotype and Allele Distributions of miR-423 rs8067576 Polymorphism in Two Groups

Genetic Models	Control, % (n = 300)	Case, % (n = 300)	χ2	OR (95% CI)	*P*
TT	28 (9.33)	15 (5.00)	–	1	–
TA	106 (35.33)	93 (31.00)	2.009	1.638 (0.825–3.253)	0.156
AA	166 (55.33)	192 (64.00)	5.403	2.159 (1.115–4.180)	0.020
Dominant					
TT	28 (9.33)	15 (5.00)	–	1	–
TA/AA	272 (90.67)	285 (95.00)	4.234	1.956 (1.022–3.742)	0.040
Recessive					
TT+TA	134 (44.67)	108 (36.00)	–	1	–
AA	166 (55.33)	192 (64.00)	4.682	1.435 (1.034–1.992)	0.030
Alleles					
T	162 (27.00)	123 (20.50)	–	1	–
A	438 (73.00)	477 (79.50)	6.999	1.434 (1.097–1.875)	0.008
p^{HWE}	0.073				

Abbreviations: HWE, Hardy-Weinberg Equilibrium; OR, odd ration; CI, confidence interval.

Correlation of miR-423 rs8067576 Polymorphism with Clinical Characteristics of TNBC Patients

Table 3 summarizes the comparison results of key clinicopathological features for TNBC patients with different miR-423 rs8067576 genotypes. The data indicated that cases harboring the AA allele exhibited a higher prevalence of tumors >5 cm ($P = 0.034$), lymph-node involvement ($P = 0.023$), and higher stage (III–IV; $P = 0.001$), implying that this variant is indicative of an exacerbated disease manifestation. In contrast, no comparable correlations were disclosed for age or menopausal status ($P > 0.05$).

Table 3 Correlation of miR-423 rs8067576 Polymorphism with Clinical Characteristics of TNBC Patients

Parameters	TT/TA (n = 108)	AA (n = 192)	*P* Value
Age, year			0.085
>50	76	116	
≤50	32	76	
Menopause			0.442
No	69	114	
Yes	39	78	
Histological subtype			0.415
Ductal	89	165	
Lobular	19	27	
Tumor size, cm			0.034
≤5	84	127	
>5	24	65	
LNM			0.023
Negative	73	104	
Positive	35	88	
TNM			0.001
I–II	79	104	
III–IV	29	88	

Abbreviations: TNBC, triple-negative breast cancer; TNM, tumor-node-metastasis; LNM, lymph node metastasis.

Correlation of miR-423 rs8067576 Polymorphism with Disease Progression and Prognosis of TNBC Patients

To delineate the independent prognostic determinants influencing the RFS of patients, in this study, all TNBC patients were grouped according to their RFS status. The K-M plot evidenced that cases harboring the AA genotype exhibited inferior RFS than those with TT/TA genotype (log Rank P = 0.0015; Figure 1B), suggesting AA genotype to be indicative of a tumor-promoting role. Beyond that, potential clinical indicators were included in the multivariate Cox regression model to analyze the independent correlation of miR-423 rs8067576 with TNBC (Table 4 and Figure 1A). The results showed that after adjusting for other confounding factors, tumor size (HR = 1.529, 95% CI = 1.008–2.321; P = 0.046), TNM (HR = 1.527, 95% CI = 1.033–2.255; P = 0.034) and rs8067576 polymorphism (HR = 1.984, 95% CI = 1.185–3.323; P = 0.009) retained independently significant correlation with RFS. The data demonstrated that rs8067576 AA genotype conferred a higher risk of RFS for TNBC patients.

During the follow-up period, a total of 72 deaths were recorded. The K-M curve results denoted that the survival period of patients with the AA genotype was significantly shortened compared to TT/TA genotype carriers (log Rank P = 0.0006; Figure 1C). The Cox regression results implied that after adjusting for confounding variables, the Cox proportional hazards model further confirmed that TNM stage (HR = 1.743, 95% CI = 1.090–2.787; P = 0.020) and rs8067576 (HR = 2.922, 95% CI = 1.467–5.820; P = 0.002) were both independent prognostic factors for TNBC (Table 5 and Figure 1D).

Impacts of rs8067576 Polymorphism on the Quantitative Expression Profiles of miR-423 and Tumor Cell Function

Upon Drosha/Dicer cleavage, pre-miR-423 is processed into two mature strands, namely miR-423-3p and miR-423-5p. To assess the impact of rs8067576 polymorphism on mature miR-423 expression, isogenic plasmids encoding the T or A allele were individually transfected into one normal MCF-10A cell line and two TNBC cell lines (BT-20 and MDA-MB-231). As shown in Figure 2A, allele-specific quantification revealed that rs8067576-A allele selectively boosted miR-423-5p expression in non-malignant MCF-10A (1.15 fold, $P < 0.05$), whereas miR-423-3p remained unchanged regardless of T allele ($P > 0.05$). Notably, in both BT-20 (2.11 fold; Figure 2B) and MDA-MB-231 (2.46 fold; Figure 2C) cell lines, miR-423-5p abundance was remarkably raised by rs8067576-A allele (all $P < 0.001$), while leaving the miR-423-3p altered slightly (1.14 fold and 1.23 fold, respectively). Clinically, such a correlation was also observed in TNBC patients. In control participants, both miR-423-3p and miR-423-5p levels did not differ significantly among different rs8067576 genotype carriers (Figure 2D and E). For TNBC patients, AA genotype carriers owned the highest miR-423-5p levels, while TT carriers had the lowest one (Figure 2E). Collectively, these data supported that rs8067576-A allele broadly amplifies miR-423-5p output in TNBC background.

Functionally, cell proliferation, migration and invasion in BT-20 and MDA-MB-231 were tracked to evaluate the role of rs8067576 polymorphism in cell growth. CCK-8 assay showed that both cells harboring rs8067576-A allele proliferated faster than T allele, which were all superior to the empty vector control (Figure 2F and G). In addition, the Transwell assay results suggested that rs8067576-A allele significantly heightens the motility of tumor cells. MCF-7 and MDA-MB-231 cells carrying rs8067576-A allele displayed remarkable increases in migrated cells than the T allele counterparts (Figure 2H). Similarly, invasive ability was also heightened in the two cells, which likewise surpassed empty-vector controls (Figure 2I). Thus, these findings establish that A-allele-driven miR-423-5p overexpression robustly enhances the migratory and invasive capacity of TNBC cells.

Functional Annotation and Pathway Enrichment of the Target Genes

By integrating the prediction results from the three databases, namely TargetScan, miRDB and micro-T, a total of 83 intersecting target genes of miR-423-5p were identified (Figure 3A). GO enrichment analysis revealed that the functions of the 83 intersecting target genes were significantly concentrated in three major dimensions (Figure 3B). BP focused on intracellular signal transduction and positive regulation of transcription mediated by RNA polymerase II. CC were centered around in chromatin and cytoplasm. At the MF level, it was mainly enriched in GTPase binding, ATP binding

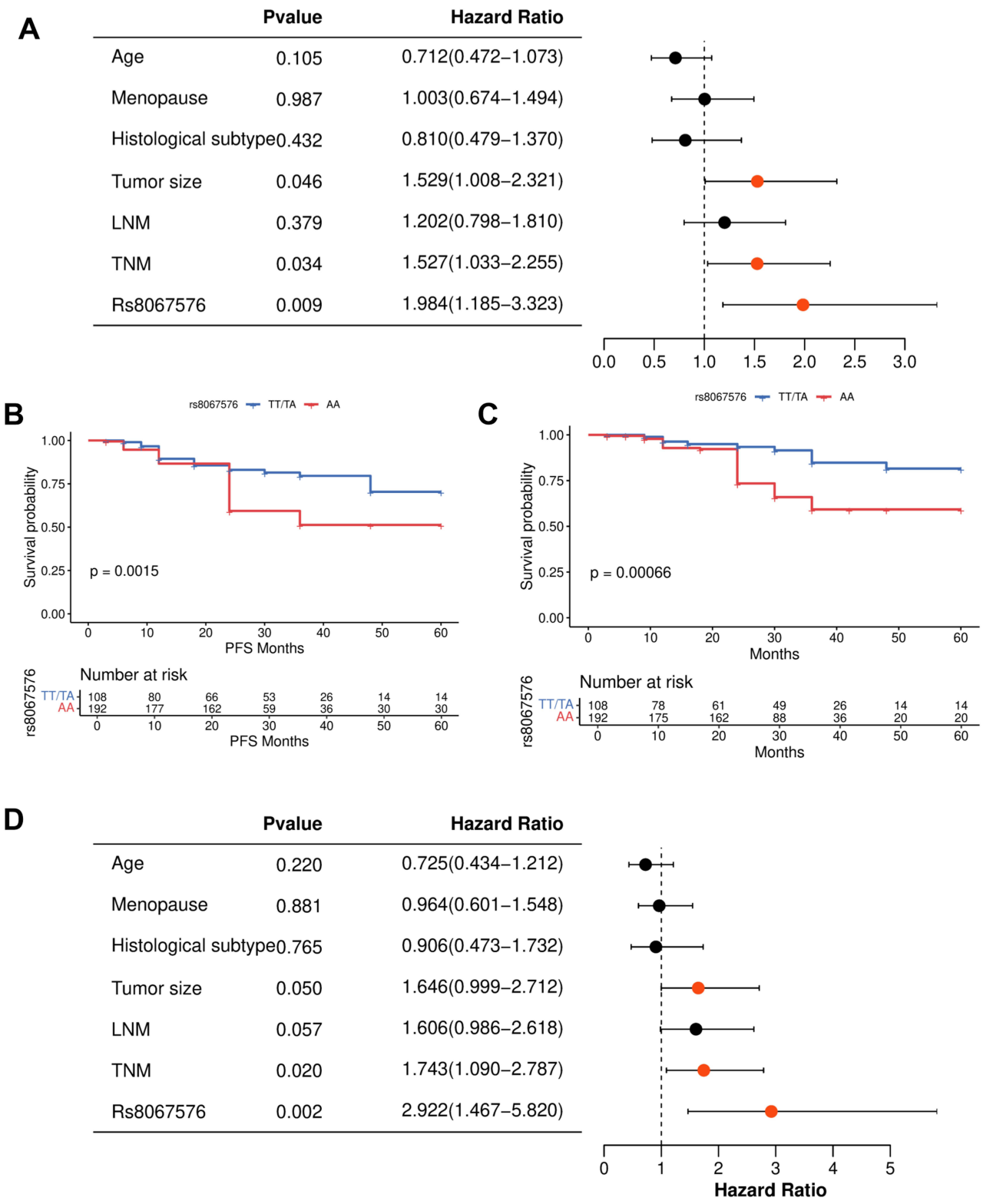


Figure 1 Correlation of miR-423 rs8067576 polymorphism with disease progression and prognosis of TNBC patients. (**A**). Multivariate Cox regression analysis results of independent factors related to relapse-free survival (RFS) of TNBC patients. (**B**). KM plot of RFS for TNBC patients with different rs8067576 genotypes. (**C**). KM plot of 5-year overall survival for TNBC patients with different rs8067576 genotypes. (**D**). Multivariate Cox regression analysis results of independent factors related to 5-year overall survival of TNBC patients.

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Table 4 Correlation of miR-423 rs8067576 Polymorphism with RFS of TNBC Patients

Feature	HR	95% CI	*P* Value
Age	0.712	0.472–1.073	0.105
Menopause	1.003	0.674–1.494	0.987
Histological subtype	0.810	0.479–1.370	0.432
Tumor size	1.529	1.008–2.321	0.046
LNM	1.202	0.798–1.810	0.379
TNM	1.527	1.033–2.255	0.034
Rs8067576	1.984	1.185–3.323	0.009

Abbreviations: TNBC, triple-negative breast cancer; LNM, lymph node metastasis; TNM, tumor-node-metastasis; RFS, Relapse-free survival; HR, hazard ratios; CI, confidence intervals.

Table 5 Correlation of miR-423 rs8067576 Polymorphism with Overall Survival of TNBC Patients

Feature	HR	95% CI	*P* Value
Age	0.725	0.434–1.212	0.220
Menopause	0.964	0.601–1.548	0.881
Histological subtype	0.906	0.473–1.732	0.765
Tumor size	1.646	0.999–2.712	0.050
LNM	1.606	0.986–2.618	0.057
TNM	1.743	1.090–2.787	0.020
Rs8067576	2.922	1.467–5.820	0.002

Abbreviations: TNBC, triple-negative breast cancer; LNM, lymph node metastasis; TNM, tumor-node-metastasis; HR, hazard ratios; CI, confidence intervals.

activities and protein binding. The KEGG pathway analysis revealed that the Wnt and Ras signaling pathways were the core pathways with significant enrichment of target genes (Figure 3C).

Target Gene Verification

We further selected representative target genes highly associated with the Wnt and Ras pathways and TNBC for validation. Ago2-RIP experiments demonstrated significant enrichment of RNF43 (Figure 4A and B) and PAK3 (Figure 4D and E) in both BT20 and MDA-MB-231 cell lines, with RNF43 exhibiting a markedly higher enrichment fold than the other two targets. Luciferase reporter assays confirmed that miR-423 directly targets and binds to the 3'UTR regions of the two candidate genes RNF43 (Figure 4C) and PAK3 (Figure 4F), establishing them as direct target genes. The evidence suggested miR-423 exhibits relatively higher in vivo binding efficiency and recruitment into the miRISC complex with RNF43.

Discussion

SNPs are the most common form of genetic variation in the genome and have been widely studied for their association with breast cancer (BC). Numerous studies have shown that specific SNPs are strongly linked to an increased susceptibility to BC.[13] Certain SNPs may act as prognostic markers for BC, offering promising clinical utility in early detection and risk stratification.[14] In-depth investigation of the functions and mechanisms behind SNPs promises to generate fresh concepts and powerful instruments for advancing precision oncology in BC.[15] This study revealed a significant difference in the genotype distribution of the miR-423 rs8067576 polymorphism between tumor patients and controls. And the AA genotype was positively associated with increased tumor size, LNM and advanced TNM stage. Additionally, carriers of

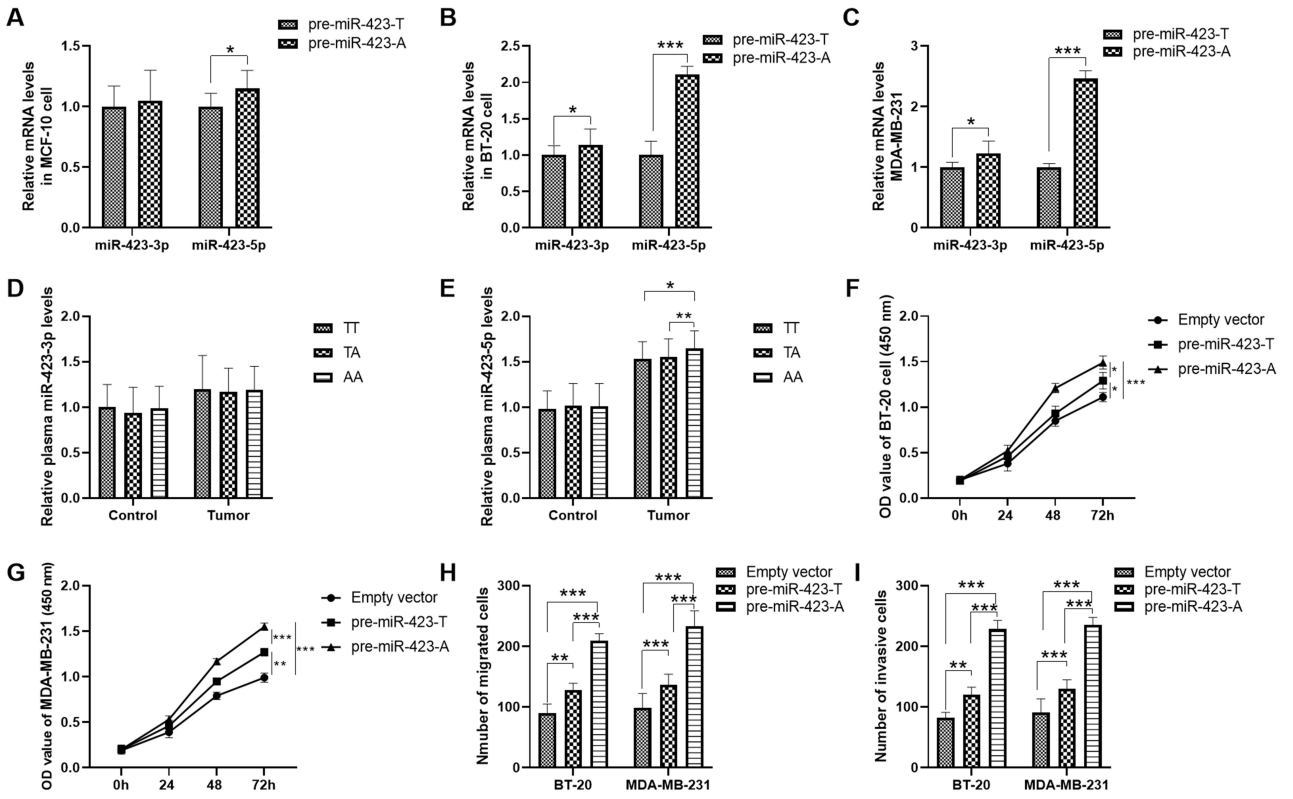

Figure 2 Impacts of rs8067576 polymorphism on the expression profiles of miR-423 and tumor cell function. (**A–C**). miR-423-3p or miR-423-5p levels in MCF-10, BT-20 and MDA-MB-231 cells after transfecting rs8067576-T or A allele. (**D** and **E**). miR-423-3p or miR-423-5p levels in the plasma of TNBC patients with different rs8067576 genotypes. (**F** and **G**). Cell viability of BT-20 and MDA-MB-231 cell lines after transfecting rs8067576-T or A allele. (**H**). Migrated cell number of BT-20 and MDA-MB-231 cell lines after transfecting rs8067576-T or A allele. (**I**). Invasive cell number of BT-20 and MDA-MB-231 cell lines after transfecting rs8067576-T or A allele. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

this allele displayed markedly reduced RFS and 5-year overall survival. In the clinical setting, individuals harboring AA genotype held a conspicuous rise in miR-423-5p levels compared with patients bearing alternative genotypes. Cell-based assays revealed that introducing this SNP allele into tumor cells robustly boosted tumor-cell proliferation, motility, and invasiveness relative to the alternate allele. Subsequent target prediction and pathway enrichment flagged Wnt and Ras signaling as the principal downstream circuits of miR-423-5p.

SNPs in embedded miRNAs act as discreet but potent modulators in the initiation and progression of TNBC.[16,17] By perturbing the stability of pri- and pre-miRNAs, altering the cellular abundance of mature miRNAs, or weakening the thermodynamic fit between a miRNA and its target mRNAs, these subtle variants rewrite post-transcriptional circuitry. Ultimately, the resulting imbalance may silence tumor-suppressor genes or unleash oncogenes, gradually steering otherwise normal mammary epithelium toward malignant transformation and sustained progression.[18] For example, rs2910164, a C/G polymorphism nestled in the pre-miR-146a hairpin, heightens breast-cancer risk by elevating both miR-146a-3p and miR-146a-5p, thereby fueling proliferation, migration and invasion while restraining apoptosis.[13] Likewise, the A allele of rs6505162 boosts miR-423 expression, endowing tumor cells with enhanced proliferative and metastatic potential and conferring cisplatin resistance.[19] Here, the genotypic spectrum of miR-423 rs8067576 was sharply skewed between TNBC patients and healthy controls, and individuals harboring the AA genotype displayed markedly higher miR-423-5p levels, supporting the notion that rs8067576 serves as a susceptibility locus of TNBC susceptibility by tuning the abundance of miR-423-5p. Literature has established that SNPs not only mirror tumor aggressiveness but also foretell dismal prognosis. In HER2-positive breast cancer, the rs2910164 C allele is linked to significantly shortened survival.[20] We now show that carriers of the rs8067576 AA genotype harbor larger tumors, exhibit more frequent lymph-node metastasis, and present at advanced TNM stages, implying that this locus may propel malignant progression by disrupting miRNA expression.

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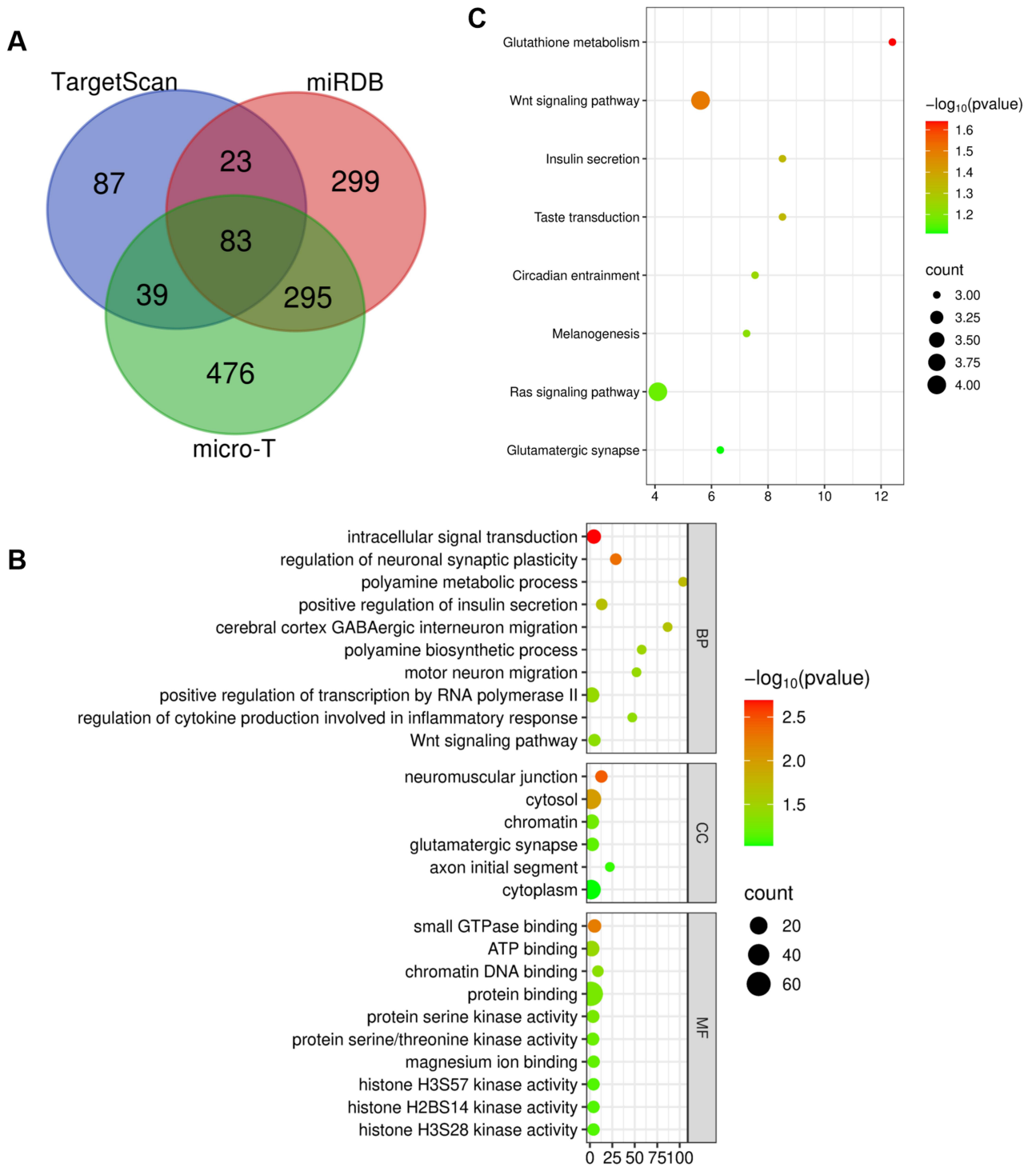

Figure 3 Functional annotation and pathway enrichment of the target genes. (**A**). Target genes of miR-423-5p based on three databases, namely TargetScan, miRDB and micro-T. (**B**). GO enrichment analysis of 83 intersecting target genes. (**C**). KEGG pathway enrichment analysis of 83 intersecting target genes.

miR-423 stands out among microRNAs for its conspicuous overexpression in BC, it is emerging as a pivotal orchestrator of tumorigenesis, progression, and therapeutic response.[21–23] Quantitative analyses reveal that both clinical specimens and established cancer cell models display a pronounced surge in miR-423 compared with their normal counterparts.[19] Translational cohort studies further demonstrate that escalating miR-423 levels parallel advancing TNM stage, nodal burden, and distant dissemination, underscoring its promise as a biomarker and therapeutic target. Moreover,

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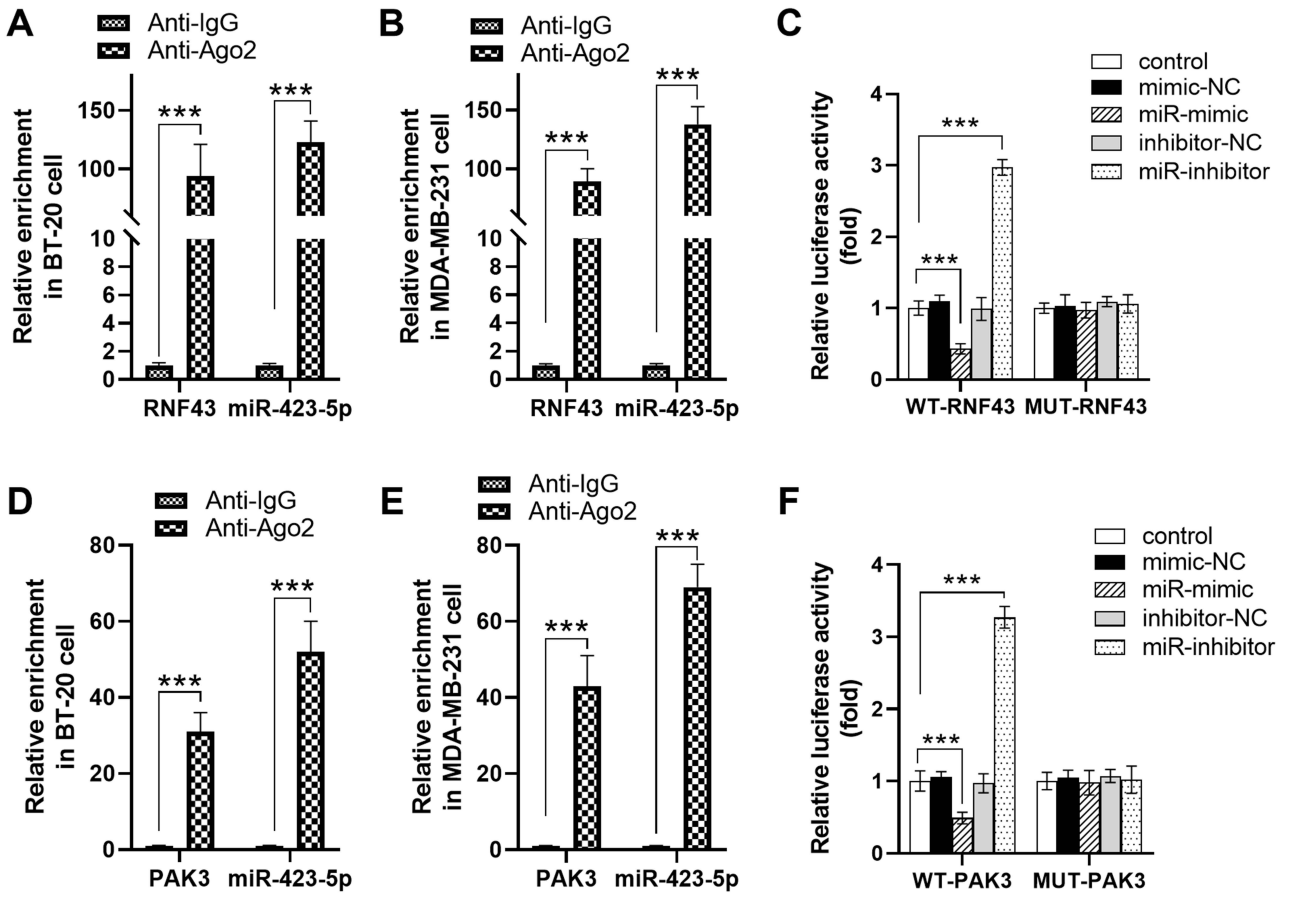

Figure 4 Target gene verification. (**A** and **B**). RIP assay of RNF43 in BT20 and MDA-MB-231 cell lines. **C**. Luciferase reporter assay of RNF43 in 293T cell. (**D** and **E**). RIP assay of PAK3 in BT20 and MDA-MB-231 cell lines. (**F**). Luciferase reporter assay of PAK3 in 293T cell. *** $P < 0.001$.

miR-423 fuels BC aggressiveness via several molecular mechanisms. It can amplify proliferative signaling, bolster motility and invasiveness, and render cells refractory to chemotherapy. Mechanistically, by down-regulating TNIP2, miR-423 ensures the activation of NF-κB signaling and thereby sharply escalates the migratory and invasive capacity of tumor cells.[24] Our data established that the rs8067576 AA genotype independently predicted unfavorable outcomes, conferring both an abbreviated median RFS and a markedly lower 5-year overall survival probability. Mechanistically, patients harboring rs8067576 AA genotype displayed markedly elevated miR-423-5p expression compared to carriers of other genotypes. This robust correlation strongly indicates that elevated miR-423-5p expression may be a key molecular mechanism underpinning the unfavorable prognosis of patients with the rs8067576 AA genotype. Moreover, it furnishes novel clinical evidence endorsing miR-423-5p as a promising biomarker for tumor-prognosis evaluation, further supporting its important role in orchestrating tumor progression and prognostic regulation. Further cell-based assays revealed that tumor cells harboring the rs8067576-A allele displayed markedly enhanced proliferation, migration, and invasion compared to T allele. These data connoted that rs8067576 may contribute to TNBC risk by mediating miR-423-5p expression and, consequently, the oncogenic behavior of BC cells.

This study, through the prediction of miRNA downstream target genes and the enrichment of signaling pathways, found that the Wnt and Ras signaling pathways are the core pathways. Previous studies have well established that the Wnt and Ras signaling pathways play crucial roles in tumor initiation and progression.[25,26] Studies have shown that the overexpression of Wnt-1 and β-catenin contributes to the expansion of breast cancer stem cells and tumor heterogeneity.[27] Abnormal activation of the Wnt signaling pathway is instrumental in breast cancer resistance to chemotherapy and radiotherapy.[28] In breast cancer, the overexpression of Ras and its downstream effector molecules, such as EGFR and ERK, fosters tumor proliferation and metastasis.[29] Our findings reveal that miR-423-5p may sculpt the

malignant phenotype of TNBC cells by fine-tuning Wnt- and Ras-pathway target genes, offering fresh mechanistic insight into tumor pathogenesis. We further selected representative target genes highly associated with the Wnt and Ras pathways and TNBC for validation. All three target genes were validated as direct targets of miR-423 through dual verification by luciferase and RIP assays. Ring finger protein 43 (RNF43) exhibited the highest enrichment level in the RIP assay, indicating its high binding affinity and basal expression level. RNF43 is an E3 ubiquitin ligase that plays a crucial role in various cancers. In TNBC, RNF43 functions as a tumor suppressor gene, with its low expression associated with aggressive disease progression.[30] RNF43 acts as a negative regulator of the Wnt signaling pathway. Functional experiments demonstrate that overexpression of RNF43 inhibits the proliferation and migration capabilities of TNBC cells.[30] These findings suggested that among the multiple direct targets regulated by miR-423, RNF43 serves as the dominant core target gene, while the target gene PAK3 may exert synergistic regulatory roles, collectively mediating downstream biological effects. However, their regulatory mechanisms and functions require further investigation.

The primary limitations of this study are as follows. The sample size was limited, and the findings require further validation in larger-scale, multicenter cohort studies. As a case-control study, this research only demonstrates associations and cannot establish causality. Furthermore, due to the lack of in-depth mechanistic investigations, the relevant biological pathways and molecular regulatory mechanisms require further exploration through subsequent experiments.

Conclusion

Collectively, this study established miR-423 rs8067576 as a susceptibility locus for TNBC and linked its carriage to earlier relapse and shorter 5-year survival. At the mechanistic level, rs8067576 boosted miR-423-5p expression, thereby fueling tumor-cell proliferation, motility, and invasiveness. Through potent crosstalk within the Wnt–Ras nexus, rs8067576 may propel oncogenic evolution. These findings offer a fresh vantage point for risk prediction, prognostication, and targeted intervention in TNBC.

Data Sharing Statement

Corresponding authors may provide data and materials.

Ethics Approval and Consent to Participate

The study protocol was approved by The Ethics Committee of Traditional Chinese Medical Hospital of Wenling affiliated to Zhejiang Chinese Medical University (Date: 2017.11.15/NoYXLL-2017-235). All procedures performed in studies involving human participants were in accordance with the 1964 Helsinki Declaration and later versions. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Consent for Publication

All patients provided written informed consent.

Author Contributions

HL P, ZX H, AP L, MY L, YX L, Y L and JY C conducted the experiment and analyzed the data. HLP and ZX H wrote the manuscript. JY C revised the manuscript. 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

There is no conflict of interest in this study.

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