A Novel ATM Antisense Transcript ATM-AS Positively Regulates ATM Expression in Normal and Breast Cancer Cells

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[Abstract] Objective: The ataxia telangiectasia mutated (ATM) gene is a master regulator in cellular DNA damage response. The dysregulation of ATM expression is frequent in breast cancer, and is known to be involved in the carcinogenesis and prognosis of cancer. However, the underlying mechanism remains unclear. The bioinformatic analysis predicted a potential antisense transcript ATM-antisense (AS) from the opposite strand of the ATM gene. The purpose of this study was to identify ATM-AS and investigate the possible effect of ATM-AS on the ATM gene regulation.

Methods: Single strand-specific RT-PCR was performed to verify the predicted antisense transcript ATM-AS within the ATM gene locus. qRT-PCR and Western blotting were used to detect the expression levels of ATM-AS and ATM in normal and breast cancer cell lines as well as in tissue samples. Luciferase reporter gene assays, biological mass spectrometry, ChIP-qPCR and RIP were used to explore the function of ATM-AS in regulating the ATM expression. Immunofluorescence and host-cell reactivation (HCR) assay were performed to evaluate the biological significance of ATM-AS in ATM-mediated DNA damage repair. Breast cancer tissue samples were used for evaluating the correlation of the ATM-AS level with the ATM expression as well as prognosis of the patients.

Results: The ATM-AS significantly upregulated the ATM gene activity by recruiting KAT5 histone acetyltransferase to the gene promoter. The reduced ATM-AS level led to the abnormal downregulation of ATM expression, and impaired the ATM-mediated DNA damage repair in normal breast cells in vitro. The ATM-AS level was positively correlated with the ATM expression in the examined breast cancer tissue samples, and the patient prognosis.

Conclusion: The present study demonstrated that ATM-AS, an antisense transcript located within the ATM gene body, is an essential positive regulator of ATM expression, and functions by mediating the binding of KAT5 to the ATM promoter. These findings uncover the novel mechanism underlying the dysregulation of the ATM gene in breast cancer, and enrich our understanding of how an antisense transcript regulates its host gene.

Key words: antisense transcript; ataxia telangiectasia mutated; ataxia telangiectasia mutated-antisense; breast cancer; KAT5

Breast cancer is the most commonly diagnosed cancer, and has a complex pathogenesis and high heterogeneity[1,2]. The identification of potentially actionable molecular features and molecular “drivers” would improve our understanding of the breast cancer tumorigenic mechanism. DNA damage response (DDR) is involved in the activation of a series of cellular activities to repair DNA lesions and maintain genomic integrity, and is critical in preventing tumorigenesis[3]. The ataxia-telangiectasia mutated (ATM) protein kinase is a master regulatory factor in the DNA double-strand break (DSB) response[4]. The mutations and dysregulation of the ATM gene have been involved in the carcinogenesis and development of breast cancer[5]. For example, loss of heterozygosity at the ATM locus has been reported in 30%–40% of breast tumors[6]. In
addition, a comprehensive meta-analysis that included 19 studies estimated a cumulative risk of 6.02% for breast cancer in heterozygous ATM mutation carriers by 50 years of age, and a cumulative risk of 32.83% by 80 years of age.

Increasing evidence has shown that the ATM expression is downregulated in breast cancer tissues, without detectable mutation or deletion of the ATM gene. The reported expression level of ATM in breast cancer tissues was approximately 50% lower than that in paracancerous tissues or benign breast disease tissues. In addition, the protein expression was lower than that in normal tissues in 75% of sporadic breast carcinomas. Furthermore, the ATM expression was reduced in lymph nodes obtained from 71% of metastatic breast carcinomas. Importantly, the ATM expression is an independent prognostic factor for breast cancer patients, since patients with a high expression of ATM have a significantly better prognosis than patients with a low expression of ATM. At present, the mechanism of ATM dysregulation in breast cancer remains unclear, regardless of its recognized impact on the development and prognosis of breast cancer. The ATM gene spans for more than 150 kb at the 11q23.1 chromosomal region, and consists of 66 exons. Our previous bioinformatics analysis predicted an antisense transcript, namely, ATM-antisense (AS), which originates from the reverse strand of the ATM gene locus.

The expression level of this potential antisense transcript was positively correlated with the ATM expression in breast cancer samples in the present study. Antisense transcripts have been increasingly recognized as important regulators due to the overlapping sense genes. The mechanisms underlying antisense transcript-mediated gene regulation include the alteration in the splicing, localization, transport and stability of the sense mRNA transcript through the formation of sense-antisense RNA duplexes, which induce transcriptional collision by misleading the transcription direction, and regulate the gene expression by binding to the promoters of the sense genes. In mammalian cells, the fraction of genes associated with antisense transcripts varies from less than 2% to more than 70% of the total genes. This implies that antisense transcript-mediated gene regulation is a common regulatory mechanism, and that this might be a part of the self-regulatory circuits that allow genes to regulate their own expression.

In the context of ATM dysregulation in breast cancer, we proposed that the ATM-AS antisense transcript might be an important regulator of ATM expression in cells. The present study aimed to determine whether and how ATM-AS regulates the ATM expression in normal and breast cancer cells. It was found that ATM-AS can regulate the ATM expression by mediating the binding of KAT5 to the ATM promoter, thereby increasing the histone acetylation and enhancing the ATM promoter activity. In the breast cancer tissue samples, the significant decrease in ATM-antisense (AS) was positively correlated with the alteration in ATM expression. These present findings indicate that ATM-AS functions as a positive regulator of ATM expression, and that the decrease in ATM-AS expression is directly correlated to the dysregulation of ATM expression in breast cancer.

1 MATERIALS AND METHODS

1.1 Cell Culture
The MCF-10A, MCF-7, MDA-MB-231 and ZR-75-1 cells were obtained from the American Type Culture Collection (ATCC, USA). MCF-10A cells were grown in DMEM-F/12 supplemented with 5% horse serum (HS) and four additional factors (0.02 μg/mL of epidermal growth factor, 10 μg/mL of insulin, 0.5 μg/mL of hydrocortisone, and 0.1 μg/mL of chloromycetin). MCF-7 cells were grown in minimal essential medium supplemented with 10% fetal bovine serum (FBS). MDA-MB-231 cells were grown in F15 supplemented with 10% cow serum (CS). ZR-75-1 cells were grown in DMEM supplemented with 10% FBS. All culture media contained 200 U/mL of penicillin and 100 μg/mL of streptomycin.

1.2 Clinical Samples
A total of 65 paired breast cancer tissues and adjacent non-tumor tissues were obtained from patients in Jiangsu Province Hospital. All patients were diagnosed with breast cancer based on the histopathologic evaluation before surgery. Clinical information, including gender, age, pathological subtype and lymph node metastasis, was collected from all patients. All collected tissue samples were immediately snap frozen in liquid nitrogen, and stored at −80°C until required. The present study was approved by the Ethics Committee of Nanjing Medical University (2018-497). All experimental methods abided by the Helsinki Declaration.

1.3 RNA Isolation and qRT-PCR Analysis
For the qRT-PCR analysis, the total RNA was isolated from cells using TRIzol Reagent (Takara, Japan), according to manufacturer’s protocol. Cytoplasmic and nuclear RNA was fractionated using the PARIS™ Kit (Life Technologies, USA). All RNA samples were treated with a DNA wiper, and the yield and quality of the RNA samples were evaluated prior to the qRT-PCR. The first-strand cDNA was synthesized from 1 μg of total RNA using a reverse transcriptase cDNA synthesis kit (Vazyme, China). Then, the resulting cDNA was analyzed by qRT-PCR using a SYBR Green PCR Kit (Vazyme, China) and the 7500 Fast real-time PCR system (Applied Biosystems,
UK). For detecting the gene expression, the results were normalized to GAPDH. The experiments were independently repeated for at least three times, in order to ensure the reproducibility of the results.

For the detection of cytoplasmic and nuclear expression, the GAPDH was amplified as an internal control. Then, β-actin and U6 were used as cytoplasmic and nuclear controls, respectively. MALAT1 was detected as a reference nuclear location for IncRNA. The comparative quantification was performed using the \(2^{-\Delta\Delta Ct}\) method. The primer sequences were, as follows: MALAT1-F: 5′-CTTAAGGCAGGCCACATTTT-3′, MALAT1-R: 5′-CCTCACAACCCCAAGACCAA-3′; U6-F: 5′-CTCGCTTCGGCAGCACA-3′, U6-R: 5′-AAGCTTAAAAGCTGCTTTG-3′; β-actin-F: 5′-ACGCTTCAAGTTTGGCGT-3′, β-actin-R: 5′-CTGGAGGAGCAATGATCTTG-3′; GAPDH-F: 5′-GCACCGTCAAGGCTGAGAAC-3′, GAPDH-R: 5′-TGTTGAAGACGCCAGTGA-3′.

1.4 Single Strand-specific RT-PCR (SSPCR)

The antisense strand-specific cDNA was obtained via first-strand reverse transcription (RT) using an antisense-specific RT primer, 5′-CTGGTAAGGGGTATGTGGTGAAGACGCCAGTCAAGGCTGAGAAC-3′, according to manufacturer’s instructions (Vazyme, China). A DNA wiper was used before the RT reaction.

1.5 Plasmids and Luciferase Reporter Assay

The expression vectors for human ATM (pcDNA3.1-ATM) were cloned from the full-length cDNA. The ATM promoter region was cloned into the pGL3-basic reporter vector that expressed the firefly luciferase for the luciferase assay. Then, the ATM-AS shRNA sequences, 5′-AGCTTAAAAGCTGCTTTG-3′; ATM-ChIP-S1 (F: 5′-ACCGCCAGTCTCAACTCGTA-3′, R: 5′-GTTATTTGGACCCGGCTATGC-3′); ATM-ChIP-S2 (F: 5′-TGGTGAAGACGCCAGTCAAGGCTGAGAAC-3′, R: 5′-TGTTGAAGACGCCAGTGA-3′), were synthesized and inserted into the pRNAT-H1.1/neo vector. For the luciferase reporter assay, cells were seeded into 12-well plates, and co-transfected with a series of plasmids on the next day, which included the firefly reporter constructs that contained the ATM gene promoters, the Renilla expressing plasmid and the firefly reporter constructs. The co-precipitated nuclear RIP RNA samples, input samples and IgG control samples were detected by qRT-PCR. The primer sequences were as follows: ATM-ChiP-S1 (F: 5′-CTGAAGCTGGGAGGC-3′, R: 5′-GTCCAATAACCTCCATCC-3′); ATM-ChiP-S2 (F: 5′-TGGTGAAGACGCCAGTCAAGGCTGAGAAC-3′, R: 5′-TGTTGAAGACGCCAGTGA-3′), were synthesized and inserted into the pRNAT-H1.1/neo vector. For the luciferase reporter assay, cells were seeded into 12-well plates, and co-transfected with a series of plasmids on the next day, which included the firefly reporter constructs that contained the ATM gene promoters, the Renilla expressing plasmid and the firefly reporter constructs.

1.6 DNA Pull Down Assay

A biotin-labeled double-stranded DNA probe within the ATM promoter region was synthesized to pull down the ATM promoter-binding proteins. The probe sequence was as follows: 5′-AGGCAGCTCTCACTCCATCTCTTGAGTAATTGAGCTTCAAGGGTGAGGAGCCAAACCCCAAGCTTCAACCAAGGGAAAACCTTTGGCCTCAAAGGTCTTCTGTCCAGCATAGCCCGTGTC

1.7 Chromatin Immunoprecipitation Assay

Chromatin Immunoprecipitation (ChIP) assay was performed using a ChIP assay kit (Millipore, USA), essentially as described by the manufacturer. A total of \(1\times10^5\) cells for each sample were fixed in 1% formaldehyde at 37°C for 10 min. Then, these cells were lysed and sonicated into 200–1000 bp fragments, and incubated with the antibody overnight at 4°C. The reversal of cross-linking was carried out at 65°C for 5 h, followed by DNA isolation. For the quantitative analysis of ChIP products, real-time PCR was carried out using the SYBR Green real-time PCR Master Mix (Vazyme, China), according to kit instructions. The primer sequences used for the experiment are listed as follows: ATM-Chip-S1 (F: 5′-ACCGCCAGTCTCAACTCGTA-3′, R: 5′-GTCCAATAACCTCCATCC-3′); ATM-Chip-S2 (F: 5′-TGGTGAAGACGCCAGTCAAGGCTGAGAAC-3′, R: 5′-TGTTGAAGACGCCAGTGA-3′), were synthesized and inserted into the pRNAT-H1.1/neo vector. For the luciferase reporter assay, cells were seeded into 12-well plates, and co-transfected with a series of plasmids on the next day, which included the firefly reporter constructs that contained the ATM gene promoters, the Renilla expressing plasmid and the firefly reporter constructs. The co-precipitated nuclear RIP RNA samples, input samples and IgG control samples were detected by qRT-PCR. The primer sequences were as follows: ATM-Chip-S1 (F: 5′-ACCGCCAGTCTCAACTCGTA-3′, R: 5′-GTCCAATAACCTCCATCC-3′); ATM-Chip-S2 (F: 5′-TGGTGAAGACGCCAGTCAAGGCTGAGAAC-3′, R: 5′-TGTTGAAGACGCCAGTGA-3′), were synthesized and inserted into the pRNAT-H1.1/neo vector. For the luciferase reporter assay, cells were seeded into 12-well plates, and co-transfected with a series of plasmids on the next day, which included the firefly reporter constructs that contained the ATM gene promoters, the Renilla expressing plasmid and the firefly reporter constructs. The co-precipitated nuclear RIP RNA samples, input samples and IgG control samples were detected by qRT-PCR. The primer sequences were as follows: ATM-Chip-S1 (F: 5′-ACCGCCAGTCTCAACTCGTA-3′, R: 5′-GTCCAATAACCTCCATCC-3′); ATM-Chip-S2 (F: 5′-TGGTGAAGACGCCAGTCAAGGCTGAGAAC-3′, R: 5′-TGTTGAAGACGCCAGTGA-3′), were synthesized and inserted into the pRNAT-H1.1/neo vector. For the luciferase reporter assay, cells were seeded into 12-well plates, and co-transfected with a series of plasmids on the next day, which included the firefly reporter constructs that contained the ATM gene promoters, the Renilla expressing plasmid and the firefly reporter constructs.
(Cell Signaling Technology, 20E3, USA), washed, and further stained with a secondary antibody for 1 h. Hoechst staining was used to identify the nuclei.

1.10 Western Blot Analysis

In order to determine the protein level of ATM, cells were collected and lysed on ice in RIPA lysis buffer that contained the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). Then, the protein from each sample was separated on 10% polyacrylamide gels. After electrophoresis, the separated proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Roche Applied Science, Germany). Afterwards, these membranes were blocked for 1 h at room temperature with 5% BSA (Boster, AR0004, China). Then, these PVDF membranes were incubated overnight with the anti-ATM (dilution 1:500; Abcam, ab32420, USA), anti-GAPDH (1:2000, Cell Signaling Technology, 5174S, USA), or anti-β-actin (1:5000, Sigma-Aldrich, USA) antibody. Subsequently, the membranes were incubated with the horse-radish peroxidase-conjugated secondary antibody at room temperature for 1 h. The signals of the bands were detected using ECL reagents.

1.11 Statistical Analysis

All experiments were repeated at least three times. All data were analyzed using the GraphPad Prism 6.0 software (GraphPad Software, USA), and expressed as mean±standard deviation (SD). Comparisons between two groups were analyzed using Student’s \( t \)-test to determine the level of significance between the control and treatment groups. The \( \chi^2 \)-test was used to determine the correlation between ATM-AS and ATM in breast cancer tissues. \( P<0.05 \) was considered statistically significant.

2 RESULTS

2.1 ATM Gene Contained a Novel Antisense Transcript Named ATM-AS

The investigators explored the possible mechanism underlying the dysregulation of the ATM gene expression by searching for antisense transcripts that may be functionally correlated to ATM regulation using the UCSC genome browser. The annotation of GRCh38/hg38 revealed a LINE-1 \( (L1) \) gene located in Intron 61 of the ATM gene. The 5'UTR of the \( L1 \) gene contained an ORF0 sequence in the antisense orientation with a potential upstream antisense promoter (ASP). Notably, several expressed sequence tags (ESTs) were also mapped to this locus (AA954560 and DB356801). These analyses imply the presence of a potential antisense transcript that originated from a novel gene located on the opposite strand of the ATM gene (fig. 1A).

Subsequently, SSPCR was performed using the predicted antisense strand-specific cDNA in the MCF-10A normal breast epithelial cell line, and the MCF-7, ZR-75-1 and MDA-MB-231 breast cancer cell lines, in order to confirm the existence of the potential antisense transcript. Sense strand-specific cDNA samples were used as negative controls. The accuracy of the detection was ensured by treating the RNAs used in these experiments with DNase, in order to remove the possible residual genomic DNA. These present results confirm the presence of a novel transcript, designated as ATM-AS, which was transcribed from the antisense strand of the ATM locus in both normal breast epithelial cells and breast cancer cells (fig. 1B).

Fig. 1 Identification of ATM-AS, a novel antisense transcript at the ATM gene locus

A: schematic representation of the positions of the ATM gene and its antisense ATM-AS. The “+” at the left side of the strand represents the positive strand; The “−” represents the negative strand. The arrows indicate the transcription direction. The different genes were labeled in different colors. The blue blocks indicate the exons of the ATM gene. The predicted L1 ASP is also indicated in the figure. Two EST transcripts, in the antisense direction and overlapping ATM-AS were labeled in the figure (AA954560 and DB356801). B: The antisense-specific RT-PCR screenings for ATM-AS used the negative strand cDNA (labeled Strand −), and these were performed for MCF-10A, MCF-7, ZR-75-1 and MDA-MB-231 cells. Sense-specific cDNA (labeled Strand +) samples were used as negative controls. ATM-AS: ataxia telangiectasia mutated-antisense
Next, it was determined whether ATM-AS was transcribed from the potential upstream antisense promoter of \( L1 \) (L1-ASP) by initially performing dual-luciferase reporter assays to evaluate the promoter activity of L1-ASP. Unexpectedly, no promoter activity was detected for the predicted ASP of the \( L1 \) gene (fig. S1A). Therefore, the investigators performed further PCR tests using primers that mapped to L1-ASP or \(-650 \) bp of the predicted TSS (fig. S1B). Using all these primers, the PCR produced the expected specific products (fig. S1C). These results indicate that ATM-AS was initiated from an unknown upstream ASP, rather than from the L1-ASP. While searching for the transcription initiation of ATM-AS, the investigators also attempted to use the 3′RACE to determine the 3′-end of ATM-AS. However, the 3′RACE failed to successfully work, because the ATM-AS gene contains multiple poly(A/T) sequences that produce truncated PCR products, and interfere with the accurate determination of the 3′-end using 3′RACE. Therefore, the investigators used the PCR walking method to determine the approximate length of the downstream extension. With 5 pairs of primers, the PCR walking successfully amplified a 6-kb continuous downstream sequence, although the AT rich sequences within ATM-AS made it difficult to continue the further walk down. These results indicated that ATM-AS was at least 6000 nt in length, and that this overlapped with the Exon60 and Exon61 of ATM in an antisense fashion. This offered the investigators the opportunity to explore the functional role of ATM-AS in the regulation of ATM gene expression.

2.2 Expression Level of ATM-AS Was Positively Correlated with ATM Expression in Breast Cancer Tissues and Prognosis of Patients with Breast Cancer

The correlation between the expression levels of ATM-AS and ATM in vitro and in vivo was determined to evaluate the functional relationship. As indicated in fig. 2A and 2B, the mRNA and protein levels of ATM were lower in the MCF-7, ZR-75-1 and MDA-MB-231 breast cancer cell lines than in the MCF-10A normal breast epithelial cell line, corresponding to the expression patterns of ATM-AS in these cell lines (fig. 2C). Furthermore, the expression levels of ATM and ATM-AS decreased by 73.8% (48/65) and 84.6% (55/65), respectively, in breast cancer tissues, when compared to those in paracancerous tissues (figs. 2D and 2E), with a significantly positive correlation (correlation coefficient=0.672, \( p<0.01 \); fig. 2F).

**Fig. 2** Expression pattern of ATM-AS and ATM in cell lines and tissues

The ATM mRNA (A) and protein (B) levels, and the ATM-AS expression level (C) were detected in the MCF-10A normal breast epithelial cell line, and MCF-7, ZR-75-1 and MDA-MB-231 breast cancer cell lines. A and C: The RNA expression levels were detected by qRT-PCR, and were presented as fold changes vs normal breast cells. ***\( p<0.001 \) vs. MCF-10A cells. B: The ATM protein levels were measured by Western blotting. The ATM (D) and ATM-AS (E) expression levels were detected in 65 breast cancer tissues and matching normal adjacent tissues. ***\( p<0.001 \) vs. MCF-10A cells. F: the correlation between the ATM-AS and ATM mRNA expression in breast cancer tissues. \( r=0.6721, p<0.0001 \) vs. MCF-10A cells. ATM-AS: ataxia telangiectasia mutated-antisense
The correlation between the ATM-AS expression and the widely used Nottingham Prognostic Index (NPI) was also analyzed to evaluate the biological significance of ATM-AS in breast cancer patients. The Nottingham grade (NG) was assigned according to the final NPI score (NG1=score of 3–5, NG2=score of 6–7, and NG3=score of 8–9), and the patients were stratified into different prognostic categories, in which NG1 was the best prognosis and NG3 was the worst prognosis. In 58 cases of breast cancer, the samples were broadly classified according to the ATM-AS expression (ATM-AS low and ATM-AS high). Then, the NPI was calculated, the pathologic stage was recorded for each individual case, and the total cases were classified into different prognostic groups. Since no case of NG1 was found, NG2 was defined as NPI low and NG3 was defined as NPI high. The cross-tabulation of NPI scores against the ATM-AS expression (table 1) revealed the significant association between NPI and ATM-AS (Chi-square=4.392, \( P \)=0.036). The proportion of patients with a high ATM-AS expression decreased from 82.1% to 56.7% as the NPI score increased, indicating that the higher ATM-AS expression level is associated with better outcome. These in vitro and in vivo data strongly suggest the possible involvement of ATM-AS in the positive regulation of ATM expression.

| NPI category | ATM-AS Low | ATM-AS High | Total |
|--------------|------------|-------------|-------|
| Low          | 5 (17.9%)  | 23 (82.1%)  | 28 (48.3%) |
| High         | 13 (43.3%) | 17 (56.7%)  | 30 (51.7%) |
| Total        | 18 (31.0%) | 40 (70.0%)  | 58 (100%) |

ATM-AS: ataxia telangiectasia mutated-antisense; NPI: Nottingham prognostic index

### 2.3 ATM-AS Up-regulated ATM Expression at Transcriptional Level

The functional role of ATM-AS by cytoplasmic and nuclear RNA fractionation was evaluated to determine the distribution of ATM-AS in MCF-10A cells that expressed high levels of ATM-AS. The qRT-PCR results revealed that ATM-AS was mainly localized in the nucleus (fig. 3A), suggesting its involvement in gene regulation. The nuclear location IncRNA MALAT1 was measured in this experiment as a reference gene. Then, the functional role of ATM-AS in the regulation of ATM expression was determined through the knockdown of ATM-AS in the normal MCF-10A cell line. The qRT-PCR and Western blotting results revealed that the knockdown of ATM-AS strikingly reduced the ATM mRNA and protein levels (fig. 3B–3D). Similar results were obtained for the MCF-7 breast cancer cell line (fig. S2).

Since ATM-AS is mainly distributed in the nucleus, dual luciferase reporter gene assays were performed to test the effect of the ATM-AS action on ATM promoter activity. As indicated in fig. 3E, the knockdown of ATM-AS significantly attenuated the ATM promoter activity in MCF-10A cells. These results indicate that ATM-AS is a positive regulator of ATM promoter activity.

#### 2.4 ATM-AS Was Required for KAT5 Acetyltransferase Binding to ATM Promoter

The investigators sought to understand how ATM-AS regulates the ATM transcription by examining the influence of ATM-AS knockdown on protein binding in the ATM promoter region. MCF-10A cells were transfected with shATM-AS or shControl. Then, two rounds of mass spectrometry (MS) were performed. In the first round, DNA pull-down assay was performed using DNA sequence S1 (fig. 4A) within the ATM promoter region as a probe. Then, the pull down proteins were separated by two-dimensional gel electrophoresis, followed by biological mass spectrometry analysis. However, the expected result was not obtained. In order to improve the detection efficiency, the strategy was changed. The MS analysis was directly performed on the pull down proteins. In the second round of MS, a total of 300 proteins were found to bind at the ATM gene promoter (table S1). Among these proteins, an acetyltransferase KAT5 strongly caught the attention of the investigators.

ChIP-qPCR was further performed to evaluate the binding of KAT5 at the ATM promoter, and the effects of ATM-AS on the binding. A total of four pairs of PCR primers were used, as shown in the schematic diagram in fig. 4A (left panel). The immunoprecipitated DNA was analyzed with three primer sets that were specific to the regions of the ATM promoter. Amplicons S1 and S2 covered the position of the DNA probe used in the previous DNA pull down experiment, and amplicon S3 was located at approximately 200 bp upstream of S1, inside the ATM promoter. A pair of primers located within the ATM gene body was also used as the negative control (S4). Consistent with the present MS data, the ChIP-qPCR revealed that at ATM promoter region S1 and S2, the binding of KAT5 to the ATM promoter sharply dropped after the knockdown of ATM-AS. However, there was no binding of KAT5 in promoter region S3, and in the internal region of the ATM gene locus S4 (fig. 4A, right panel). Furthermore, these results revealed that the KAT5 binding at the ATM promoter sharply dropped after the knockdown of ATM-AS, indicating the requirement of ATM-AS for KAT5 binding to the ATM promoter.

Next, the investigators further investigated the possible mechanism underlying the ATM-AS dependent binding of KAT5 by performing RIP assays combined with ChIP assays using the same batch of extracts obtained from MCF-10A cells, and the antibody against KAT5. As shown in fig. 4B, KAT5 bound to both the
ATM-AS transcripts and \( \text{ATM} \) promoter, suggesting that ATM-AS functions as a scaffold to mediate the binding of KAT5 to the \( \text{ATM} \) promoter.

### 2.5 Histone Acetyltransferase Activity of KAT5 Was Required for Activation of \( \text{ATM} \) Transcription

Subsequently, it was determined whether KAT5 is involved in the regulation of \( \text{ATM} \) expression. MG149, a specific KAT5 HAT inhibitor, was used to inhibit the histone acetyltransferase (HAT) activity of KAT5 in MCF-10A. Then, the change in \( \text{ATM} \) expression was examined by qRT-PCR and Western blotting. As shown in fig. 5A and 5B, the inhibition of KAT5 activity resulted in the significant decrease in mRNA and protein expression levels of \( \text{ATM} \), indicating that the \( \text{ATM} \) transcription requires the HAT activity of KAT5. The ChIP-qPCR assays, which used the antibody against H3Ac, also confirmed that the knockdown of ATM-AS reduced the H3 acetylation of the \( \text{ATM} \) promoter (fig. 5C). These data, together with the results described above, verified that the activation...
of ATM transcription required the ATM-AS-mediated binding of KAT5 to the ATM promoter to increase its histone H3 acetylation.

2.6 ATM-AS Was Required for ATM-mediated DNA Damage Repair

ATM is a key regulator of DNA DSB repair. Therefore, the investigators explored the biological function of ATM-AS in ATM-mediated DNA DSB repair using γH2AX assays in ATM-AS knockdown MCF-10A cells, or in control cells treated with bleomycin (BLM) for 24 h. As shown in fig. 6A and 6B, the γH2AX-positive signals were significantly higher in BLM-treated ATM-AS knockdown MCF-10A cells than in BLM-treated control cells. The increase in γH2AX positive cells could be completely reversed by the re-expression of ATM in cells (fig. 6B), indicating that the depletion of ATM-AS elevates the sensitivity of breast cells to the DNA breakage induced by DNA-damaging agents.

Host-cell reactivation (HCR) assays were also performed to compare the DNA repair ability of ATM-AS knockdown MCF-10A cells and control cells. The principle of HCR is presented in fig. 6C. Briefly, pGL3-promoter plasmids with firefly luciferase were pretreated with H2O2 for one hour, in order to induce DSBs before transfection, together with Renilla luciferase plasmids, into ATM-AS knockdown MCF-10A cells and control MCF-10A cells. Then, these cells were cultured for 24 h, and harvested for fluorescence detection.

The DNA repair capacity of cells was reflected by the fluorescence values. A higher fluorescence value indicated a stronger ability to repair DNA damage. Western blotting was performed to confirm the effect of the ATM-AS knockdown on the ATM expression (fig. 6D). The HCR assay revealed that the knockdown of ATM-AS significantly impaired the repair of DNA breakage by cells. The repair efficiency was approximately 50% lower in ATM-AS knockdown MCF-10A cells than in control cells, while the overexpression of ATM in ATM-AS knockdown cells completely restored the ability for DNA repair (fig. 6E). These data strongly indicate that the ATM-AS antisense transcript functions as an important regulator for maintaining DNA repair through the positive regulation of ATM expression in breast cells.

3 DISCUSSION

The findings presented in the present study identified ATM-AS as a novel natural ATM antisense transcript. ATM-AS was able to positively regulate the ATM gene expression in normal breast cells by recruiting KAT5 to the ATM promoter. The knockdown of ATM-AS significantly decreased the KAT5-mediated H3 acetylation, which in turn suppressed the ATM expression induced by DNA damage, and the efficiency of DNA damage repair. Correspondingly, the ATM-AS expression level was positively correlated with the ATM expression in breast cancer tissues, and the prognosis of patients with breast cancer.

ATM is an important cell cycle checkpoint kinase that plays a critical role in the cellular response to DNA damage, and in maintaining genome stability. The mutations and dysregulation of ATM are well-known to be involved in carcinogenesis and the development of breast cancer. However, the mechanism underlying the abnormal downregulation of ATM expression remains unclear. The present finding that ATM can be regulated by an antisense transcript located within the gene itself provides a valuable clue for understanding the dysregulation of ATM in breast cancer cells.
ATM-induced DNA damage repair is attenuated by ATM-AS depletion

A: cell immunofluorescence (×100) analysis of the effect of ATM-AS depletion on DNA damage in MCF-10A cells. The cells were treated with shATM-AS or combined with ATM overexpression. DNA double strand breaks were induced with BLM. The immunofluorescence staining of γH2AX was measured as a marker of DSB in cells. These cells were stained with Hoechst dye to determine the cell number.

B: analysis of γH2AX-positive cells in (A). *P<0.05.

C: graphic representation of the dual luciferase reporter system used to determine the capacity for cell damage repair. D: MCF-10A cells were transfected with shATM-AS, or in combination with the ATM overexpression, followed by the transfection with the DNA damage dual luciferase reporter system. The ATM protein expression was measured in the experiment.

E: analysis of DNA repair efficiency, according to the fluorescence values of the luciferase reporter system. **P<0.01. ATM: ataxia telangiectasia mutated; BLM: bleomycin; γH2AX: the phosphorylated form of histone H2A.X
In recent years, a variety of roles have been found for natural antisense transcripts (NATs) in the regulation of gene activity\(^{[16]}\). However, the determination on how these work remains not well-understood. Three models were proposed. The first model involved the base pairing between the cis-natural antisense transcript (cis-NAT) and its complementary transcript\(^{[13]}\), the second model involved the guidance of methylation complexes and/or histone-modifying complexes to the promoter regions of the sense transcript by reverse transcript to inhibit the expression of the gene\(^{[19–21]}\), and the third model involved transcriptional collision, and this has gained favor based on recent experimental evidence\(^{[22]}\). In the present study, the newly identified ATM-AS regulator was a cis-NAT that has a transcript complementarity to ATM transcripts. However, the recombinase polymerase amplification (RPA) assays performed in the present study revealed that the ATM sense and ATM-AS antisense transcripts were unable to form a duplex RNA, regardless of the complementarity (fig. S3). Furthermore, ATM-AS and ATM were not located in the head-to-head position required for the antisense transcript to function in transcriptional collision. Moreover, ATM-AS was a positive regulator, rather than a negative regulator, of ATM expression. For these reasons, the possibility that ATM-AS regulates the ATM gene through complementary transcript knockdown or transcriptional collision can be ruled out.

Conversely, the RIP and ChIP assays performed in the present study revealed that KAT5 simultaneously binds to ATM-AS and the ATM promoter, and that these bindings increase in response to the DNA damage in normal breast cells, leading to the increase in ATM expression. Therefore, a reasonable conclusion is that ATM-AS regulates ATM gene activation by providing a scaffold or guide for the recruitment of KAT5 to the ATM promoter. ATM-AS is required for increasing and maintaining the ATM expression level in the DNA damage response of breast tissue cells. Consistent with this view, the present γH2AX and HCR assays revealed that the knockdown of ATM-AS significantly impaired the DNA breakage repair, and increased the sensitivity of cells to the DNA damage agent BLM. Given the important role of ATM in suppressing breast cancer, these present findings suggest that ATM-AS has a specific function in suppressing breast cancer. Considering ATM-AS as a newly discovered antisense transcript, our understanding of the function and underlying mechanism of ATM-AS remains at its infancy. Hence, further exploring its breast cancer suppressor function in relation to ATM is necessary and valuable for understanding the pathogenesis of breast cancer.

KAT5, which is also known as Tip60, belongs to the MYST family of HATs that function to increase the accessibility of DNA by acetylating histones to upregulate the gene expression, and regulate protein functions by acetylating non-histone proteins\(^{[23, 24]}\). Furthermore, KAT5 is essential for the activation of the ATM kinase activity at the post-translational level\(^{[25]}\). Upon DNA damage, the HAT activity of KAT5 is upregulated, and KAT5 forms a complex with ATM to directly acetylate ATM. This acetylation is required for the subsequent activation of ATM kinase activity.

The findings presented in the present study reveal the essential role for KAT5 in the regulation of the ATM gene at the transcriptional level. KAT5 can bind to the ATM promoter in an ATM-AS dependent manner to upregulate the ATM promoter activity by histone acetylation, as a DNA damage response. Therefore, KAT5 is essential not only for activating the ATM kinase activity, but also for activating the ATM gene activity in DNA damage response. Any factors that interfere with the ATM-AS-mediated KAT binding on the ATM gene promoter can result in the abnormal reduction in gene expression.

The ATM-AS sequence is more than 6000 nt in length, and overlaps at least the Exon60 and Exon61 of the ATM gene. This was confirmed as an essential regulator for KAT5-mediated ATM gene activation, but the complete ATM-AS sequence has not been identified. Therefore, the present study could not completely rule out the possibility that ATM-AS regulates the ATM gene expression through some mechanism, other than by the recruitment of KAT5 to the ATM promoter. Further efforts to identify the complete sequence of ATM-AS through more sensitive RNA-seq are required, and these would be helpful in testing alternative mechanisms.

In summary, the results presented in the present study identified a new antisense transcript ATM-AS located within the ATM gene. ATM-AS positively regulates the ATM gene activity by recruiting the KAT5 HAT to the gene promoter. Reductions in the ATM-AS level led to the abnormal downregulation of ATM expression, and impaired the subsequent ATM-mediated repair of DNA damage in normal breast cells. Furthermore, the ATM-AS level is positively correlated with the ATM expression in breast cancer tissues, and is associated with the better outcomes of patients with breast cancer. The present study provides novel insight into the dysregulation of the ATM gene in breast cancer, and enriches our understanding of the regulation mechanisms that involve antisense transcripts.

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Conflict of Interest Statement

The authors declare that they have no competing interests.

Author Xiao HAN is a member of the Editorial Board for Current Medical Science. The paper was handled by other editors and has undergone rigorous peer review process. Author Xiao HAN was not involved in the journal’s review of, or decision related to, this manuscript.

REFERENCES

1 Sung H, Ferlay J, Siegel RL, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin, 2021,71(3):209-249
2 Harbeck N, Gnant M. Breast cancer. Lancet, 2017,389(10074):1134-1150
3 O’Connor MJ. Targeting the DNA Damage Response in Cancer. Mol Cell, 2015,60(4):547-560
4 Zgheib O, Huyen Y, DiTullio RA, Jr., et al. ATM signaling and 53BP1. Radiother Oncol, 2005,76(2):119-122
5 Stagni V, Manni I, Oropallo V, et al. ATM kinase sustains HER2 tumorigenicity in breast cancer. Nat Commun, 2015,6:6886
6 Rio PG, Permin D, Bay JO, et al. Loss of heterozygosity of BRCA1, BRCA2 and ATM genes in sporadic invasive ductal breast carcinoma. Int J Oncol, 1998,13(4):849-853
7 Marabeli M, Cheng SC, Parmigiani G. Penetration of ATM Gene Mutations in Breast Cancer: A Meta-Analysis of Different Measures of Risk. Genet Epidemiol, 2016,40(5):425-431
8 Ye C, Cai Q, Dai Q, et al. Expression patterns of the ATM gene in mammary tissues and their associations with breast cancer survival. Cancer, 2007,109(9):1729-1735
9 Angele S, Treilleux I, Bremond A, et al. Altered expression of DNA double-strand break detection and repair proteins in breast carcinomas. Histopathology, 2003,43(4):347-353
10 Kairouz R, Clarke RA, Marr PJ, et al. ATM protein synthesis patterns in sporadic breast cancer. Mol Pathol, 1999,52(5):252-256
11 Feng X, Li H, Kornaga EN, et al. Low Ki67/high ATM protein expression in malignant tumors predicts favorable prognosis in a retrospective study of early stage hormone receptor positive breast cancer. Oncotarget, 2016,7(52):85798-85812
12 Feng X, Li H, Dean M, et al. Low ATM protein expression in malignant tumor as well as cancer-associated stroma are independent prognostic factors in a retrospective study of early-stage hormone-negative breast cancer. Breast Cancer Res, 2015,17:65
13 Mahmoudi S, Henriksson S, Corcoran M, et al. Wrap53, a natural p53 antisense transcript required for p53 induction upon DNA damage. Mol Cell, 2009,33(4):462-471
14 d’Ydewalle C, Ramos DM, Pyles NJ, et al. The Antisense Transcript SMN-AS1 Regulates SMN Expression and Is a Novel Therapeutic Target for Spinal Muscular Atrophy. Neuron, 2017,93(1):66-79
15 Huang B, Song JH, Cheng Y, et al. Long non-coding antisense RNA KRT7-AS is activated in gastric cancers and supports cancer cell progression by increasing KRT7 expression. Oncogene, 2016,35(37):4927-4936
16 Faghhi MA, Wahlestedt C. Regulatory roles of natural antisense transcripts. Nat Rev Mol Cell Biol, 2009,10(9):637-643
17 Pelechano V, Steinmetz LM. Gene regulation by antisense transcription. Nat Rev Genet, 2013,14(12):880-893
18 He Y, Vogelstein B, Velculescu VE, et al. The antisense transcriptome of human cells. Science, 2008,322(5909):1855-1857
19 Tufarelli C, Stanley JAS, Garrick D, et al. Transcription of antisense RNA leading to gene silencing and methylation as a novel cause of human genetic disease. Nat Genet, 2003,34(2):157-165
20 Morris KV, Santoso S, Turner AM, et al. Bidirectional transcription directs both transcriptional gene activation and suppression in human cells. PLoS Genet, 2008,4(11):e1000258
21 Bernstein E, Allis CD. RNA meets chromatin. Genes Dev, 2005,9(14):1635-1655
22 Shearwin KE, Callen BP, Egan JB. Transcriptional interference—a crash course. Trends Genet, 2005,21(6):339-345
23 Squatrito M, Gorrini C, Amati B. Tip60 in DNA damage response and growth control: many tricks in one HAT. Trends Cell Biol, 2006,16(9):433-442
24 Gobbiati AH, Kamei MA. Tip60: updates. J Appl Genet, 2018,59(2):161-168
25 Sun Y, Jiang X, Chen S, et al. A role for the Tip60 histone acetyltransferase in the acetylation and activation of ATM. Proc Natl Acad Sci USA, 2005,102(37):13182-13187

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