Estrogen Receptor α Regulates ATM Expression through miRNAs in Breast Cancer

Xiaojing Guo¹,², Chunying Yang², Xiaolong Qian¹, Ting Lei¹, Yaqing Li¹, Haifa Shen³, Li Fu¹, and Bo Xu²,⁴

Abstract

Purpose: Estrogen receptor α (ERα) is an essential element regulating mammary gland development and it contributes to breast cancer development and progression. Most of the ER-negative breast cancers display more aggressive clinical behaviors and are resistant to antiestrogen therapies. In addition, many ER-negative tumors show insensitivity to many chemotherapeutic drugs and radiotherapy, although mechanisms underlying this phenotype are less clear.

Experimental Design: We conducted immunohistochemistry on 296 cases of breast cancer tissues using a variety of antibodies. On the basis of the clinical data, we conducted siRNA knockdown to study the role of ERα on ATM expression in breast cancer cell lines. Furthermore, we used antisense oligonucleotides against micro RNAs (miRNA) or miRNA overexpression plasmids to study the role of miR-18a and -106a on ATM expression. Finally we used in situ hybridization to assess miR-18a and -106a expression in breast cancer tissues.

Results: We found that in ER-negative breast cancer tissues, expression of the ATM kinase, a critical DNA damage-response protein, is aberrantly upregulated. We also found that the locoregional recurrence rate after radiotherapy positively correlates with ATM expression. On the cellular level, we showed that ERα, but not ERβ, negatively regulates ATM expression. Furthermore, we identified that ERα activates miR-18a and -106a to downregulate ATM expression. We also showed that miR-18a and -106a were significantly underexpressed in ER-negative breast cancer tissues.

Conclusions: We reveal a novel mechanism involving ERα and miR-18a and -106a regulation of ATM in breast cancer. Clin Cancer Res; 1–9. ©2013 AACR.

Introduction

Approximately 30% of patients with breast cancer do not express estrogen receptor α (ERα; ref.1). ERα-negative tumors are a part of the triple-negative breast cancer (TNBC), a subset of the breast cancer population, which typically is associated with aggressive clinical phenotypes and poor prognosis (2). For example, ER-negative breast cancers are not responsive to antiestrogen therapies. In addition, many ER-negative patients show less sensitivity to many DNA damaging agents (3), except in some cases, such as with a Her2-positive status, patients showed variable sensitivity to chemotherapeutic drugs such as anthracycline (4, 5). At the cellular level, ERα regulates a variety of processes in mammary gland development and it also contributes to the development and progression of breast cancer (6). By binding to estradiol (E2), ERα mediates transcription by interacting directly to specific estrogen-response elements located in the promoter or enhancer region of target genes. In addition, ERα may indirectly associate with nuclear proteins such as AP1 and SP1 to stimulate transcription (7). Recent data have also shown that ERα plays a critical role in regulating (either suppress or stimulate) micro RNAs (miRNA) to control protein expression (8). Aberrant miRNA expression has been implicated in estrogen-related breast and endometrial cancers in the clinical setting (9). It has been reported that several phosphorylation events are required for a variety of cell-specific functions of ERα (10). Despite the extensive studies on ERα function in regulation of transcription, the functional mechanism driving most of ER-negative breast cancer cells resistant to many chemotherapeutic agents as well as radiotherapy is less clear.

Cellular sensitivity to DNA damaging agents such as radiotherapy and radiomimetic drugs are regulated by a cascade of DNA damage response proteins. Among them is...
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Translational Relevance
Most of the estrogen receptor α (ERα)—negative breast tumors are resistant to many DNA-damaging agents, although mechanisms underlying this phenotype are less clear. In this report, we show in cellular models and clinical samples that ERα negatively regulates expression of the ATM kinase through miR-18a and -106a. These observations highlight a novel mechanism linking ERα and ATM, which explains the resistant phenotype of ER-negative cancers to chemotherapy and radiotherapy. As ER-negative breast cancers are more aggressive and unresponsive to antiestrogen therapies, other targeted therapies are urgently needed. Because ATM expression is upregulated in ER-negative cancers, ATM might represent an interesting drug target in these tumors. Using specific ATM inhibitors might achieve more clinical benefits as this might specifically increase tumor sensitivities to many of the chemotherapeutic drugs as well as radiotherapy.

Materials and Methods
Cell culture and materials
Breast cancer cell lines MCF-7 and MDA-MB-231, obtained from American Type Culture Collection (ATCC) were maintained in Dulbecco’s modified Eagle medium containing 10% FBS (Invitrogen) and 50 mg/mL of penicillin/streptomycin (Invitrogen) in humidified 37°C chambers with 5% CO2. β-Estradiol was purchased from Sigma-Aldrich. Cell line authentication was originally done with 5% CO2.

Antibodies
Rabbit anti-ATM and mouse anti-phospho-ATM (S1981p) antibodies were purchased from Cell Signaling. Mouse anti-ATM, mouse anti-ERα and rabbit anti-ERβ antibodies were purchased from Abcam. All horseradish peroxidase-conjugated secondary antibodies and β-actin antibody were obtained from Santa Cruz Biotechnology.

Plasmids, siRNAs, and transfection
miRNA overexpression plasmids (pCMV-miR18a, 106a, 101, and 421) and ATM-3’s untranslated region (UTR) plasmid were purchased from Origene, ERα plasmid (pEGFP-C1-ERα) was purchased from Addgene. Plasmids were transfected into cells with Fugene HD Reagents. Control, ERα, ERβ, and ATM siRNAs were purchased from Santa Cruz Biotechnology and transfected into cells by Oligofectamine reagent (Invitrogen) according to the manufacturer’s instruction. Forty-eight hours after transfection, cells were analyzed. miRNA antisense oligos against has-miR18a, 106a, 101, and 421 were synthesized from Integrated DNA Technologies. Scrambled sequences were used as negative controls. All the oligos were transfected into cells also using the oligofectamine reagent. The sequences of all the oligos are listed in the following table.

<table>
<thead>
<tr>
<th>miRNA Antisense</th>
<th>Scrambled</th>
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<td></td>
</tr>
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<td>miR-101 5'ttcagttacacgtatcagtagta3' 5'agaacagactctttagtc3'</td>
<td></td>
</tr>
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<td>miR-106a 5'acctgacaggttagcacttt3' 5'ctacctccgatcgaaatgcc3'</td>
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</tr>
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<td>miR-421 5'cgcctattagttgctga3' 5'gggcctattacattccg3'</td>
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</table>

Luciferase reporter assay
MDA-MB-231 cells were seeded in triplicates in 48-well plates. Luciferase reporter plasmids (ATM 3’UTR or pMir-Target vector; Origene) were cotransfected with 50 nmol/L control, miR-18a, miR-106a plasmids, or both. Cells were harvested 48 hours after transfection and lysed for luciferase assays by a Dual-Glo Assay Kit (Promega) according to the manufacturer’s protocol.

Human breast cancer tissue samples
Paraffin-embedded materials from 296 invasive patients with breast cancer with operable breast cancer treated between 01/01/2003–31/05/2003 were provided by the Department of Breast Pathology and Research Laboratory, Cancer Hospital of Tianjin Medical University (Tianjin, China), with the approval of Tianjin Medical University Institutional Review Board. All cases were female with 29 to 75 years of age (the mean age is 51.8 years). All patients were treated either with modified radical mastectomy (n = 176) or breast-conserving therapy (n = 120). Locoregional radiotherapy was administered to the patients underwent with the breast-conserving therapy. The outcome of radiotherapy was evaluated by locoregional recurrence with a follow-up period from 9 to 96 (mean 74.63) months.
Immunohistochemistry

Immunohistochemistry (IHC) using the avidin–biotin–immunoperoxidase technique was conducted for ER and ATM in the 296 cases of clinical samples. Sections of formalin-fixed tissues from all cases were conducted using a standard protocol. Briefly, 4 μm tissue sections on coated slides were heated for antigen retrieval, pretreated with a 3% solution of hydrogen peroxide for 5 to 10 minutes, rinsed and incubated with 10% normal goat serum as a blocking agent. The sections were then incubated sequentially with the primary antibody (ER in a 1:100 dilution and ATM in a 1:200 dilution), a biotinylated secondary antibody, and avidin–peroxidase conjugate. All steps were preceded by rinsing of sections with PBS (pH 7.6). The chromogen was 3,3′-diaminobenzidine. The immunoreaction for ER and ATM in the nucleus of tumor cells was evaluated independently by 2 experienced pathologists. ER was determined positive if finding of ≥1% of tumor cell nuclei were immunoreactive and ATM was scored as previously described (13). (−) = No positive cells, (+) = 1% to 10% of the cells stained, (+++) = 11% to 50% of the cells stained, and (++++) = 51% to 100% of the cells stained.

Western blot analysis

Cell lysates were obtained by treatment with the lysis buffer (Thermo Scientific Pierce) containing the protease inhibitor cocktail (Roche) and phosphatase I and II inhibitors (Sigma), and the protein concentration was determined using the Bradford method (Bio-Rad). Equal volumes of cell lysates were loaded into 4% to 15% Mini-protean TGX precast gels (Bio-Rad) for electrophoresis. Proteins were then transferred from gels to the nitrocellulose membrane (Bio-Rad). Following incubation with 5% of non-fat milk (LabScientific) for 30 minutes, the slides were heated for antigen retrieval, pretreated with a 3% hydrogen peroxide for 5 to 10 minutes, rinsed slides were incubated with the hybridization mix and hybridized for 1 hour at 50°C to 60°C. The slides were then subsequently incubated with the blocking solution (15 minutes at room temperature), the anti-DIG reagent (60 minutes at room temperature), and with AP substrate (2 hours at 30°C). The sections were counter stained with nuclear fast red (Vector Laboratories). The stained sections were then scored for expression of has-miR18a and 106a miRNAs under the microscopy (Olympus). The sections were evaluated independently by 2 experienced pathologists according to the percentage of stained cells (18), with less than 20% of the cells stained was designated low expression of miRNA (+) and more than 20% of the cells stained was designated as high expression of miRNA (++)

Statistical analysis

Data were analyzed by χ² test, Student t test, and Pearson correlation test and P values ≤0.05 were considered significant.

Results

ATM expression is elevated in ER-negative breast cancer tissues

We recently reported ATM hyperactivation in breast cancer primary tissues with lymph node metastasis (13). When we further analyzed this set of the clinical data, we surprisingly found that ER-negative breast cancer tissues have a much higher expression level of ATM. In a total of 296 samples, we found that the ATM expression level was negatively correlated with ER status (Fig. 1; Table 1; P < 0.001, χ² test). However, the ER status showed less correlation with the active form of ATM—pS1981-ATM (P = 0.155, χ² test, Table 1). As these observations were contradictory to the data reported in a previous publication by Tommiska and colleagues (19), we repeated the IHC experiment and the analysis done by 2 independent pathologists confirmed our original observation.

Detection of miRNA expression by in situ hybridization in human breast cancer tissues

The miRCURY LNA miRNA ISH Kit and miRNA detection has-miR18a, 106a, and control probes were purchased from Exiqon. The miRNAs in situ hybridization was carried out in the 296 cases of human breast cancer samples. Briefly, breast cancer tissue slides were deparaffinized and incubated with proteinase-K for 10 minutes at 37°C. After dehydration, slides were incubated with the hybridization mix and hybridized for 1 hour at 50°C to 60°C. The slides were then

Real-time PCR with the SYBR green detection was conducted using an ABI Prism 7700 thermocycler with fluorescence detection (Applied Biosystems). The thermal cycling conditions were composed of 50°C for 2 minutes followed by an initial denaturation step at 95°C for 10 minutes, 45 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. The experiments were carried out in duplicate for each data point. The relative quantification in gene expression was determined using the 2−ΔΔCt method (17). Using this method, we obtained the fold changes in gene expression normalized to an internal control gene GAPDH.
A retrospective chart review of the 296 cases of breast cancer was also conducted. We focused on 120 cases who received breast-conserving surgery followed by curative radiotherapy to assess the clinical relevance of the ATM expression with patient responses to radiotherapy. Our data showed that the local recurrence rate after radiotherapy correlated with ER status (Table 2, \( P = 0.012 \)) and ATM expression (Table 2, \( P = 0.008 \); Pearson correlation test). Together, these results prompted us to further study a potential connection of ER and ATM expression.

**ER\(_\alpha\), but not ER\(_\beta\), negatively regulates ATM expression in breast cancer cells**

We hypothesized that ER might inhibit ATM expression. To test this notion, we first used an ER-positive breast cancer cell-line MCF-7. We transiently knocked down ER\(_\alpha\) (Fig. 2A) or ER\(_\beta\) (Fig. 2B) in these cells. Interestingly, we found that ATM expression levels significantly increased in ER\(_\alpha\) knockdown cells (Fig. 2A). On the contrary, ER\(_\beta\) knockdown did not show an effect on ATM expression (Fig. 2B). We also assessed ionizing radiation-induced ATM activation using the phospho-ATM Serine 1981 antibody in cells in ER\(_\alpha\) or ER\(_\beta\) knockdown cells. Unlike the effect of ER\(_\alpha\) on the basal ATM expression, ionizing radiation-induced ATM activation was unaffected by either ER\(_\alpha\) or ER\(_\beta\) knockdown. This observation is consistent with the IHC data shown in Table 1.

We then overexpressed ER\(_\alpha\) in the ER-negative breast cancer cell-line MDA-MB-231 and found that ATM expression was reduced with ER\(_\alpha\) reintroduction (Fig. 2C). On the contrary, ER\(_\alpha\) expression was not affected when ATM was knocked down by siRNA in MCF-7 cells (Fig. 2D). Together, these data indicate that ER\(_\alpha\) functions as an upstream component of the signaling network that can negatively regulate ATM expression. We also measured the ATM mRNA level in MCF-7 cells when ER\(_\alpha\) was knocked-down, however, we did not observe a change in ATM mRNA (Supplementary Fig. S1), indicating that ER\(_\alpha\) regulation of ATM is not at the transcriptional level.

**ER\(_\alpha\) regulates ATM expression through miR-18a and miR-106a**

We suspected that ER\(_\alpha\) regulation of ATM might be through ER\(_\alpha\)-regulated miRNAs. To test this notion, we

### Table 1. Correlation of ER status with ATM expression in breast cancer tissues

<table>
<thead>
<tr>
<th>ER</th>
<th>ATM Negative</th>
<th>ATM Positive</th>
<th>( P^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>16</td>
<td>55</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>+</td>
<td>23</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>++</td>
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</tr>
<tr>
<td>+++</td>
<td>30</td>
<td>13</td>
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<tr>
<td>pS1981-ATM</td>
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<td>28</td>
<td>66</td>
</tr>
<tr>
<td>+</td>
<td>24</td>
<td>50</td>
<td>0.155</td>
</tr>
<tr>
<td>++</td>
<td>15</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>+++</td>
<td>28</td>
<td>38</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) \chi^2 test.

### Table 2. Correlation of ER status and ATM expression with locoregional recurrence after breast-conserving surgery and radiotherapy

<table>
<thead>
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<th>Local recurrence</th>
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<th>No</th>
<th>( P^a )</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
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<tr>
<td>Positive</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>–</td>
<td>2</td>
<td>24</td>
<td></td>
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<td>+</td>
<td>3</td>
<td>35</td>
<td>0.008</td>
</tr>
<tr>
<td>++</td>
<td>5</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>+++</td>
<td>8</td>
<td>17</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Pearson correlation test.
searched the available database containing the ERα-regulated miRNA signature. There are 19 identified miRNAs that can be transcriptionally activated by ERα (8). We explored the possibility that some of these miRNAs might regulate ATM expression. Using the TargetScan program, we searched the 3'UTR region of the ATM gene for miRNA binding motifs. We found that approximately 7 nucleotides at the 5' end of 3 miRNAs (miR-18a, -101, and -106a) showed complementary sequences to the 3'UTR of the ATM gene (Supplementary Fig. S2). To test whether these miRNAs indeed regulate ATM expression, we designed antisense oligonucleotides against each of the 3 miRNAs. As miR-421 was previously shown to regulate ATM expression (20), we also included the antisense oligonucleotide against miR-421 as the positive control. These oligos were transfected into MCF-7 cells. As shown in Fig. 3A, we found that inhibiting miR-18a and miR-106a expression resulted in a significant increase of ATM expression, similar to the effect of inhibiting miR-421. However, knocking down miR-101 showed an opposite effect as ATM expression was reduced in antisense miR-101 expressing cells. Moreover, simultaneously knocking-down miR-18a and -106a by antisense miRNAs resulted in a more marked increase in ATM expression (Fig. 3B).

To further test ATM expression regulated by miR-18a and -106a, we used pCMV retroviral-based expression constructs containing vector or miR-18a, -106a, -101, or -421. We infected MDA-MB-231 cells with these constructs and measured the change of ATM expression. We found that overexpression of miR-18a or -106a reduced ATM expression (Fig. 4A). However, expressing miR-101 did not show any effects on ATM expression. Furthermore, we found that coexpression of miR-18a and -106a resulted in a more significant reduction of ATM expression (Fig. 4B). We also used a luciferase reporter assay following cotransfection of the luciferase reporter plasmid with miR-18a or miR-106a into MDA-MB-231 cells. Significant reductions in the luciferase activity from the reporter construct containing ATM 3'UTR were observed in the presence of miR-18a or miR-106a (Supplementary Fig. S3). Taken together, we conclude that ERα mediates ATM expression through miR-18a and -106a in breast cancer cells.

miR-18a and -106a are underexpressed in ER-negative breast cancer tissues

To test the clinical connection of ER, miR-18a and -106a, and ATM expression, we conducted in situ hybridization in the 296 cases of breast cancer tissues. We found that both miRNAs showed significant reduced expression levels in ER-negative tissues (P = 0.008 and 0.033 for miR-18a and miR-106a, respectively; Supplementary Fig. S4; Table 3).
Furthermore, expression of miR-18a and -106a negatively correlated with ATM expression ($P < 0.001$ and $P = 0.001$ for miR-18a and miR-106a, respectively; Table 3). However, we did not observe any correlation of expression miR-18a and 106a with Serine 1981 phosphorylated form of ATM (Table 3). Combining with the data presented in Fig. 1 and Table 1 on the negative correlation of ER status and ATM expression, these observations further show the involvement of miR-18a and -106a in ERα-dependent ATM regulation.

**Discussion**

In this report, we show in both cellular models and clinical samples that ERα negatively regulates expression of the ATM kinase. ATM is critical to determine the cellular survival to DNA damaging agents. In ER-negative breast cancer tissues, we observed aberrant upregulation of ATM expression. We elucidated that ERα suppressed ATM expression through regulating miR-18a and 106a. These observations highlight a novel mechanism linking ERα and ATM, which might explain the resistant
phenotype of many ER-negative cancers to DNA damaging agents.

It is noted that there is discrepancy of our observations with a previous report that ATM expression is reduced in Brca1/2–deficient and TNBC cells (19). Although it is not clear why the results differ, one possible explanation is that the clinical samples we used were all from patients with sporadic breast cancer and in the previous article, most of the non-BRCA tumors samples were from patients with familial breast cancer.

It is interesting to observe that despite its regulation on ATM expression, ERα does not affect ATM activation in response to ionizing radiation. Although it is widely accepted that ATM Serine 1981 phosphorylation is a marker of ATM activation in response to ionizing radiation, it is much less clear to what extent ATM activation might affect the radiation response in human tissues. Furthermore, ATM Serine 1981 phosphorylation induced by 6Gy of ionizing radiation is not directly relevant to the clinical responsiveness to radiotherapy. Our data shown in Fig. 1 has suggested that ATM expression seems to be more relevant to the therapeutic outcome of breast cancer radiotherapy. It is possible that other mechanisms of ATM regulation (such as the alterations of ATM expression due...
to ERα and its regulated miRNAs) contribute to the outcome of locoregional recurrence. However, we do not rule out that other factors regulated directly by ERα or regulated through ERα-induced miRNAs are important for locoregional recurrence.

Unlike on ATM expression, ERα can directly regulate the DNA damage-responsive proteins. For example, a recent report showed that ERα-activated DNA-PKcs, a family member of the PI-3–like kinases (21). In addition, p53 can be a direct target of ERα (22). However, how this regulation is associated with the clinical responsiveness of ER-negative tumors to DNA damaging agents are not clear. These regulatory cascades might be related to antiestrogen resistance as hyperactivation of the PI3K pathway, the most frequently mutated pathway in breast cancer, promotes antiestrogen resistance (23).

During our study, the link of miR-18a-ATM has been reported (24). Our data provide a more comprehensive picture of miRNA regulation of ATM expression in breast cancer tissues. In addition to miR-18a, we found miR-106a also regulates the expression of ATM. More interestingly, deletion of both miRNAs results in a more significant effect on ATM expression, highlighting that multiple miRNAs regulates ATM in breast cancer tissues. As miRNAs serve as fine-tuning regulators of protein expression, we do not rule out that other mechanisms might regulate ATM expression in breast cancer tissues. For example, ATM promoter methylation shown in locally advanced breast cancer (25) might contribute low ATM expression in ER-positive tissues.

As most of the ER-negative breast cancers are more aggressive and unresponsive to antiestrogen therapies, other targeted therapies are urgently needed. Because ATM expression is upregulated in ER-negative cancers, ATM might represent a more plausible drug target in these tumors. Using specific ATM inhibitors might achieve more clinical benefits as this might specifically increase tumor sensitivities to many of the chemotherapeutic drugs as well as radiotherapy.

In summary, we identified the signaling transduction pathway involving ERα-miR18a and 106a-ATM that provides an explanation of chemo- and radioresistance in many ER-negative breast cancers.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: X. Guo, C. Yang, L. Fu, B. Xu
Development of methodology: X. Guo, C. Yang, L. Fu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Guo, C. Yang, T. Lei, Y. Li
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Guo, C. Yang, X. Qian, B. Xu
Writing, review, and/or revision of the manuscript: X. Guo, C. Yang, H. Shen, L. Fu, B. Xu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Yang
Study supervision: C. Yang, L. Fu, B. Xu

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Table 3. Correlation of miR-18a and -106a with ER status and ATM expression in breast cancer tissues

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<th>miR106a</th>
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aχ² test.
References

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