ATM down-regulation is associated with poor prognosis in sporadic breast carcinomas


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Background: Ataxia telangiectasia-mutated (ATM) gene down-expression has been reported in sporadic breast carcinomas (BC); however, the prognostic value and mechanisms of ATM deregulation remain unclear.

Patients and methods: ATM and miRNAs (miR-26a, miR-26b, miR-203, miR-421, miR-664, miR-576-5p and miR-18a) expression levels were evaluated by quantitative real-time PCR (RT-qPCR) in 52 BC and 3 normal breast samples. ATM protein expression was assessed by immunohistochemistry in 968 BC and 35 adjacent normal breast tissues. ATM copy number alteration was detected by array comparative genomic hybridization (aCGH) in 42 tumours.

Results: Low ATM levels were associated with tumour grade. Absence of ATM protein expression was associated with distant metastasis (P < 0.001), reduced disease-free survival (DFS, P < 0.001) and cancer-specific survival (CSS, P < 0.001). Multivariate analysis indicated ATM protein expression as an independent prognostic marker for DFS (P = 0.001, HR = 0.579) and CSS (P = 0.001, HR = 0.554). ATM copy number loss was detected in 12% of tumours and associated with lower mRNA levels. miR-421 over-expression was detected in 36.5% of cases which exhibit lower ATM transcript levels (P = 0.075, r = -0.249).

Conclusions: The data suggest that ATM protein expression is an independent prognostic marker in sporadic BC. Gene copy number loss and miR-421 over-expression may be involved in ATM deregulation in BC.

Key words: ataxia-telangiectasia-mutated (ATM) gene, breast cancer, copy number alteration, microRNA, prognostic marker

Introduction

Ataxia telangiectasia-mutated (ATM) is a tumour suppressor gene that encodes a serine/threonine kinase involved in DNA repair. ATM activates DNA damage response pathways, mainly due to double-strand breaks. These harmful DNA lesions lead to genomic rearrangements and chromosomal instability contributing to tumorigenesis [1].

ATM mutations cause Ataxia Telangiectasia (AT), an autosomal-recessive neurodegenerative disorder characterized by cerebellar ataxia associated with immunodeficiency, cancer predisposition, radiosensitivity, insulin-resistant diabetes and premature ageing. In addition to its pivotal role in DNA damage response, ATM is involved in cell cycle control, apoptosis, gene regulation, oxidative stress and telomere maintenance and is deregulated in several malignancies, including BC [2, 3]. Higher risk of developing BC and other tumours was first reported in mothers of patients with AT. Subsequently, ATM mutation was associated with moderate risk to BC development [4, 5]. Although several ATM mutations have been described, they are rare and frequently associated with hereditary BC [6].

In sporadic BC, ATM transcript and protein down-regulation has been reported [7–9], though the mechanisms involved in ATM deregulation are still unknown. Allelic loss has been proposed as one of the mechanisms for ATM gene inactivation in sporadic BC cases [10], but the second allele inactivation mechanism is still unclear [4]. ATM epigenetic silencing mediated by CpG island methylation has been proposed, but contradictory results have been reported. ATM hypermethylation was found in 18 of 33 (78%) locally advanced BC [7]. Nonetheless, recent reports have indicated that ATM promoter hypermethylation is not associated with ATM under-expression in BC [11–13], indicating the existence of an alternative ATM regulatory mechanism.

A post-transcriptional ATM regulation mechanism mediated by microRNAs has been reported in neuroblastoma [14], glioma [15] and BC [16]. MicroRNAs are small non-coding RNAs that...
regulate gene expression through degradation or inhibition of mRNA translation. MicroRNAs have been associated with cell cycle regulation, growth, apoptosis, differentiation and stress response. It is well known that microRNA deregulation plays a role in tumour development and progression by modulating oncogenic or tumour-suppressor pathways [17].

Clinical significance of ATM deregulation in prognosis of BC patients is limited and controversial. This study aimed to assess ATM gene and protein expression in sporadic BC and their association with clinical outcome. In addition, we sought to investigate ATM gene regulatory mechanisms, focussing on DNA copy number alterations and miRNA expression.

Materials and Methods

Patients

Ductal invasive BC samples (N = 978) were obtained from Amaral Carvalho Hospital and AC Camargo Hospital. Clinical and histopathological data are summarized in supplementary Table S1, available at Annals of Oncology online.

Fifty-two macro-dissected fresh tumour tissues (>80% of tumour cells) were obtained for RNA and DNA extraction. Transcript analyses were performed in 52 samples and array-CGH (aCGH) in 42 of 52 samples.

Protein analysis included 42 formalin fixed paraffin-embedded (FFPE) BC (also evaluated for miRNA analysis) in conventional slides and 926 tumours arranged in four tissue microarrays (TMAs). In 35 of 42 tumours, adjacent normal breast tissues were also evaluated (conventional slides).

Six normal breast tissues from healthy individuals subjected to breast reduction surgery were obtained as controls. Three macro-dissected fresh tissues (>80% of mammary epithelial cells) and three FFPE samples were used for transcript and Immunohistochemistry (IHC) analyses, respectively.

Patients were followed prospectively with a mean follow-up of 81.9 ± 35.1 months. All patients were untreated before sample collection. Of the 978 patients, 793 have received surgery as the primary treatment; 185 and 160 received neoadjuvant chemotherapy and radiotherapy, respectively; 339 were treated by adjuvant hormonal therapy, 585 by radiotherapy and 404 by chemotherapy.

The patients were advised of the procedures and provided written informed consent. The Human Research Ethics Committee of both institutions approved the study.

ATM Gene Expression by Reverse Transcription-Quantitative PCR

Total RNA was extracted from using Trizol reagent (Invitrogen Life Technologies, Inc., Carlsbad, CA). cDNA synthesis and amplification are described in supplementary material, available at Annals of Oncology online. Primer sequences are provided in supplementary Table S2, available at Annals of Oncology online.

ATM and p53 Protein Expression by Immunohistochemistry

IHC for ATM and p53 was performed as detailed in supplementary material, available at Annals of Oncology online.

ATM Gene Copy Number Alteration by aCGH

Gene copy number alteration was assessed by aCGH using Agilent Human CGH 180K Oligo Microarrays according to the manufacturer’s recommendations (detailed in supplementary material, available at Annals of Oncology online).

MicroRNA Expression by RT-qPCR

Five algorithms were used to select the top ranked candidates for ATM regulation. The selected microRNAs were: hsa-miR-421, hsa-miR-26a, hsa-miR-26b, hsa-miR-203, hsa-miR-664, hsa-miR-576-5p and hsa-miR-18a. Target and reference primer sequences are provided in supplementary Table S3 and S4, available at Annals of Oncology online, respectively. Reverse transcription-quantitative PCR (RT-qPCR) for microRNA analysis is given in supplementary material, available at Annals of Oncology online.

data analysis

Kruskal–Wallis or Mann–Whitney tests was applied to compare ATM transcript levels and clinicopathological characteristics or ATM gene copy number alteration. Chi-square test and Fisher’s exact test were used to determine the association between the categorical variables. Bonferroni correction for multiple comparisons was applied to adjust the P-values. Spearman’s test was performed for correlation analyses between ATM and microRNA levels. Disease-free survival (DFS) and cancer-specific survival (CSS) were calculated using the Kaplan–Meier method. The end-point for CSS and DFS analysis was restricted to distant metastasis development and death due to BC, respectively. Patients with stage IV (N = 72), treated with neoadjuvant therapy (N = 170) and missing follow-up information (N = 47), were excluded from these analyses. Significant variables detected in univariate analyses were included in multivariate Cox proportional hazard regression model. GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA) and SPSS version 17.0 (SPSS; Chicago, IL) were used for statistical analysis.

Results

ATM Expression and Association with Clinical and Histopathological Variables

Decreased ATM transcript levels were detected in tumours compared with controls (P = 0.064, Figure 1A). Lower ATM mRNA levels were observed according to increased tumour grade (P = 0.005, Figure 1B). No association was found between ATM levels and other clinical and histopathological parameters. Although not significant, ATM transcript down-expression was associated with decreased DFS and CSS (data not shown).

ATM protein patterns are shown in Figure 1. Negative ATM expression was associated with tumours compared with adjacent normal breast tissues (P < 0.0001; supplementary Table S5, available at Annals of Oncology online).

ATM negativity was associated with higher grade, but it is not significant after Bonferroni correction (Table 1). Negative ATM expression was significantly associated with distant metastasis even after Bonferroni correction (P < 0.001, Table 1).

ATM-negative tumours were associated with reduced DFS (P < 0.001, Figure 1G) and CSS (P < 0.001, Figure 1H) in a large cohort of BC patients (supplementary Table S6, available at Annals of Oncology online). Significant associations were also observed between prognostic factors (T, N, clinical stage, grade, ER, PR and HER-2 status) and clinical outcome (supplementary Table S6, available at Annals of Oncology online).

Multivariate analyses showed that ATM protein status is an independent prognostic factor for DFS and CSS (Table 2). ATM-positive tumours presented lower risk of developing metastasis (HR = 0.579, P = 0.001) and cancer-specific death (HR = 0.554, P = 0.001). Tumour size and regional lymph nodes involvement were detected as independent prognostic factors for DFS, and tumour grade, tumour size and involvement of regional lymph...
Figure 1. (A) Relative expression of ATM mRNA in breast tumour samples and normal breast tissues. (B) ATM mRNA relative expression according to the tumour grade. The transcript expression values are shown in log scale and the bars indicate the median value in each group. (C–F) ATM protein expression detected by immunohistochemistry in breast tissues. Positive ATM immunostaining in normal breast tissue (C). Positive ATM adjacent normal epithelial cells (N) and ATM-negative tumour cells (arrow) (D). Negative (E) and positive (F) ATM immunostaining in breast tumour cells. Disease-free survival (G) and cancer-specific survival (H) curves according to ATM protein expression in breast cancer patients. P-values were determined by Log-rank test.
nodes for CSS (Table 2). We also evaluated if ATM stratified by p53 protein status was associated with DFS or CSS in a subset of cases (N = 221) and no association was detected (supplementary Figure S1, available at Annals of Oncology online).

evaluation of ATM gene regulatory mechanisms in BC

ATM transcript levels were compared with ATM copy number dosage obtained from another study conducted by our group using aCGH. Eleven probes span the ATM gene consisting of 66 exons. ATM locus was examined in 42 cases also evaluated for mRNA expression. Gene copy number loss was found in five cases (12%) (Figure 2A). Tumours with ATM losses showed transcript down-regulation (P = 0.013, Figure 2B). ATM protein expression was assessed in three of five cases, and two of them were negative (data not shown).

To evaluate whether ATM may be regulated by miRNAs, expression levels of predicted miRNAs (miR-26a, miR-26b, miR-203, miR-421, miR-576-5p, miR-664 and miR-18a) were detected in 52 tumours and controls also evaluated for ATM levels. Although not significant, a negative correlation was observed between the ATM and miR-421 transcript levels (r = −0.249, P = 0.075, Figure 2C, P-value was not Bonferroni-corrected). Overexpression of miR-421 was detected in 36.5% of tumours (data not shown). No significant correlation was detected between the others miRs and ATM levels (data not shown).

discussion

ATM gene deregulation has been described in sporadic BC [4], but the clinical relevance of ATM remains to be established. ATM transcript and protein down-regulation were detected in BC compared with normal breast samples, corroborating other studies [8, 9, 18–20]. We found lower levels of ATM transcript and protein expression in high-grade tumours. Ding et al. [21] showed that the combined abnormalities of ATM, BRCA1 and TP53 genes were associated with poorly differentiated BC. Additionally, ATM aberrant protein expression was associated with grade II–III tumours [9]. These findings suggest the association of ATM in more aggressive disease.
By evaluating a large cohort of patients with a long-term follow-up, an association between absence of ATM protein expression and distant metastasis was detected, suggesting that loss of ATM expression may be involved in aggressive tumour behaviour and, consequently, a worse outcome in BC patients. Survival analysis revealed that patients with ATM-negative
tumours had shorter DFS and CSS. Moreover, multivariate analysis showed that ATM protein status was an independent prognostic marker for both DFS and CSS. ATM-positive cases showed lower risk of metastasis and death due to BC. Our data suggested that ATM is an independent prognostic factor, together with tumour size and lymph nodes involvement. Ye et al. [8] evaluated ATM mRNA levels in breast tumour samples (N = 471) and showed that lower ATM transcript levels were associated with worse outcome (DFS and CSS). However, the authors did not demonstrate that ATM gene expression is an independent prognostic marker in BC. Other reports have shown association between ATM down-regulation and poor survival in BC patients with p53 wild-type tumours treated with DNA-damaging chemotherapy [22, 23]. Although with limited specificity and sensibility, TP53 missense mutations have been reported as associated with increased protein stability. Here, ATM combined with p53 status was not associated with clinical outcome in BC. Further studies should be conducted to better understand the prognostic value of ATM in the context of TP53 mutation. Thus, our data are in agreement with other studies showing that low ATM levels are associated with poor outcome in BC. However, by multivariate analysis, we found that ATM is a clinically meaningful prognostic marker in BC.

The mechanisms involved in ATM deregulation in sporadic BC are poorly understood. LOH in ATM region has been reported in sporadic BC [10, 19]. Copy number loss for the entire ATM locus detected by MLPA-analysis was recently reported in 13.6% of BC samples [23]. Similarly, we showed that ATM gene copy number loss detected in 12% of tumours was associated with transcript down-regulation. This finding suggests that ATM loss is one of the mechanisms involved in gene deregulation in BC. A recent study integrated genomic, transcriptomic and proteomic analyses on a large series of BC and identified four main tumour subtypes associated with different subsets of genetic and epigenetic abnormalities [24]. In this report, besides the disruption of p53 pathway, other pathway-inactivating events including ATM loss and MDM2 amplification were detected in BC, specifically in more aggressive luminal B subtypes [24]. Taken altogether, these data indicate that ATM gene loss and the related-pathways are important mechanisms in breast carcinogenesis.

Post-transcriptional ATM regulation can also be altered in BC. Accordingly, recent studies have indicated that ATM gene expression can be regulated by miRNAs [14–16]. ATM down-expression was detected in 38% and 61% of tumours by transcript and protein analyses, respectively. In addition, only 8 of 24 cases had both ATM mRNA and protein down-expression in paired BC samples, suggesting that post-transcriptional mechanisms such as miRNAs could be involved in gene deregulation. To address this issue, putative microRNAs regulators of ATM were investigated. Although not significant, overexpression of miR-421 was correlated with lower ATM transcript levels. It has been postulated that microRNAs can also be responsible for fine regulation of gene expression through modestly repressing a large number of mRNAs and consequently protein output [25]. Additionally, cooperation between different miRNAs in regulating target genes has been proposed [26]. Experimental validation is needed to prove the regulation of ATM by miR-421 in BC cell lines.

The involvement of miRNA in ATM expression was firstly described in HeLa and neuroblastoma cell lines, showing that miR-421 suppresses ATM expression by targeting the 3′-UTR of ATM [14]. However, ATM regulation by miRs in BC has been fairly explored. Recently, Song et al. [16] evaluated the miR-18a as a putative ATM regulatory miRNA in BC. Up-regulation of miR-18a was detected by the comparison between nine tumour cell lines and one normal epithelial cell lineage, and between 10 BC and adjacent normal tissues. ATM protein down-regulation was observed by transfection of miR-18a in BC cell lines. Conversely, ATM up-regulation was detected in these cells after miR-18a suppression. Here, miR-18a showed no correlation with ATM transcript and protein expression. Different events isolated or in cooperation can coordinate the regulation of ATM, including gene deletions, mutations, other miRNAs and promoter hypermethylation.

Taken altogether, the present study demonstrated that decreased ATM expression is associated with worse prognosis in BC. As ATM protein expression is an independent prognostic marker in BC, we suggest that ATM may be a clinically applicable marker of disease outcome. Our results also suggest that gene copy number loss and miRNA regulation may be potentially involved in ATM deregulation in sporadic BC.

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disclosure

The authors have declared no conflicts of interest.

references

Final results from the prospective phase III WSG-ARA trial: impact of adjuvant darbepoetin alfa on event-free survival in early breast cancer

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Background: WSG-ARA plus trial evaluated the effect of adjuvant darbepoetin alfa (DA) on outcome in node positive primary breast cancer (BC).

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