

ATM gene expression is associated with differentiation and angiogenesis in infiltrating breast carcinomas

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Summary. The product of the *ATM* gene, mutated in the human genetic disorder ataxia-telangiectasia (A-T) plays a key role in the detection and repair of DNA double-strand breaks. A-T is defined by progressive cerebellar ataxia, telangiectasia, sensitivity to ionising radiation and genomic instability with cancer predisposition. On the other hand, increased angiogenesis is essential for tumor growth and metastasis. The aim of this study was to investigate ATM expression in breast carcinomas and its relationship to neoangiogenesis.

Methods and Results: Fifty-two breast tumors from 51 patients, 38 of them with concomitant in situ component (CIS), were analyzed by immunohistochemistry for the expression of ATM. CD34 expression was used for the morphometric evaluation of vasculature. ATM was positive in 1 to 10% of normal epithelial cells. ATM expression was reduced in 55.8% of infiltrating carcinomas, non-reduced in 34.6%, and increased in 9.6%. Expression of ATM in CIS was similar to the infiltrating component in 71% of cases and reduced in 23.7% of them. High-grade ductal infiltrating carcinomas showed lower ATM expression than low-grade ones. Reduced ATM expression also correlated with increased microvascular area.

Conclusions: Reduced ATM expression in breast carcinomas correlated with tumor differentiation and increased microvascular parameters, supporting its role in neoangiogenesis and tumor progression in breast carcinogenesis.

Key words: ATM, DNA damage, Breast, Angiogenesis, Gene

Introduction

The human genetic disorder ataxia-telangiectasia (A-T) is a rare autosomal recessive disorder characterized by clinical manifestations that include progressive cerebellar ataxia, oculocutaneous telangiectasia, neuronal degeneration, hypersensitivity to DNA damaging agents such as ionizing radiation, premature ageing, hypogonadism, growth retardation, immunodeficiency with defects in cellular and humoral immunity, and an increased risk for cancer (Boder and Sedgwick, 1958; Chun and Gatti, 2004; Kurz and Less-Miller, 2004).

The *ATM* (A-T mutated) gene locus is located in the chromosomal region 11q22-q23. It encodes a 350-kDa protein serine/threonine kinase, member of the phosphoinositide 3-kinase (PI3-kinase)-like family (PIKK). ATM is a nuclear phosphoprotein activated in response to DNA damage (Shiloh, 2003a), with a more general role in signal transduction and in maintaining the stability of the genome (Pandita, 2002; Shiloh, 2003b). The chromosomal instability and radiosensitivity characteristic of this disease is related to the defective activation of cell cycle checkpoints. Radiation induces intermolecular autophosphorylation of Ser 1981 on ATM, causing dimer dissociation and activation (Bakkenist and Kastan, 2003), and increasing its activity as a protein kinase. ATM is also required for efficient DNA double-strand break repair, optimal phosphorylation, and activation of the p53, c-Abl, and Chk2 proteins that promote apoptosis or cell cycle arrest (Rich et al., 2000; Chen et al., 2003). Mutations causing A-T completely inactivate or eliminate the ATM protein function (Becker-Catania et al., 2000).

About half of unselected breast cancer patients have been reported to be heterozygotes for *ATM* mutations in some series (Dörk et al., 2001). Similarly, an excess of breast cancer has been reported by epidemiological studies in patients who are heterozygous for mutations in

the ATM gene and in relatives of A-T patients (Swift and Su, 1999; Thompson et al., 2005). Nevertheless, the importance of an inherited ATM mutation is controversial (Angele and Hall, 2000; Teraoka et al., 2001). Thorstenson et al., 2003 examined the role of ATM mutations in familial breast carcinomas and found a significant prevalence of ATM mutations in breast and ovarian cancer families. ATM has been proposed as a candidate tumor suppressor gene with a potential pathogenic function in breast carcinomas (Angele et al., 2003a,b) and other neoplasm's such as those of the lymphoid system (Khanna, 2000; Boultonwood, 2001; Camacho et al., 2002), rhabdomyosarcomas (Zhang, 2003), and prostate carcinomas (Angele et al., 2004a). However, the mechanisms by which deregulation of this gene contributes to these malignancies seem diverse for lymphoid and non-lymphoid tumors (Zhang et al., 2003; Chun and Gatti, 2004; Gutierrez-Enriquez et al., 2004; Lavin et al., 2004).

Angiogenesis plays an essential role in tumor development and progression in several tumor types including breast cancer (Leek, 2001; Rice and Quinn, 2002). It promotes the growth of tumors because it facilitates oxygenation and nutrient flow, and it removes metabolic waste. Angiogenesis seems to be regulated by a complex system of modulators including factors such as VEGF and HER2 (Sledge, 2002). The influence of increased angiogenesis on tumor outcome and patient prognosis has been widely studied, since it is known to be an important part of the malignant phenotype in most cancers (Hansen et al., 2000). In breast cancers, evaluation of microvasculature has been correlated with tumor evolution (Weidner et al., 1991; Kumar et al., 1999). One characteristic of the A-T syndrome is the existence of abnormal vascular development in some body territories (Di Girolami et al., 1999). Although the mechanism by which this occurs has not been elucidated, some authors propose that ATM missense variants could influence the formation of vascular abnormalities (Mauget-Faysse et al., 2003). We have thus hypothesized that levels of expression of ATM protein could be related to neoangiogenesis in breast carcinomas and that the same unknown mechanism causing this vascular abnormality in A-T patients could also be involved in local angiogenesis of breast tumors in which ATM expression is impaired.

In this study we have analyzed the expression of the ATM gene product, its relationship with clinico-pathological parameters, and its potential correlation with increased microvasculature in breast carcinomas.

Materials and methods

Patients and tissue samples

We studied 52 tumors from 51 consecutive patients with previously untreated invasive breast cancer. All tumors were surgically removed by lumpectomy or mastectomy with axillary lymphadenectomy.

Histological study

Two pathologists independently reviewed the histological material of all cases and selected the most representative sections for ulterior immunohistochemical study. One patient had bilateral infiltrating ductal carcinoma and 38 had concomitant in situ component (CIS). Recorded data were tumor size and site, histological type (42 ductal carcinomas, 4 lobular, 2 mucinous, 2 medullar, 1 papillary, and 1 adenoid cystic). Grade of invasive ductal carcinomas was evaluated according to the modified Bloom-Richardson's grading system (Elston and Ellis, 1993). Microvessel density and highest microvascularization area were also recorded. ATM expression was also related to tumor ploidy, global S phase, hormonal receptor status (ER and PR), and lymph node status. Analysis of S phase was performed with the Coulter Epics-Profile II flow cytometer on 50 micron paraffin sections, and using the Multicycle program (Phoenix Flow Systems, San Diego, CA). Normal breast samples from adjacent breast carcinoma tissue as well as four reduction mammoplasties were used as controls for immunohistochemistry.

Immunohistochemistry

For each case, a representative formalin-fixed, paraffin-embedded block tissue containing both tumor and normal breast tissue was selected. Five consecutive tissue sections from each block were cut at 3 μ m, mounted, and air-dried. One section was stained with H&E for histopathologic examination and control of the selected tumor area. The remaining ones were used for immunohistochemical stains and negative controls. Deparaffination and rehydration were performed using xylene, alcohol, and distilled water.

a. ATM Immunostaining

Slides were submitted to saponin antigen retrieval for 30 min. After blockage of endogenous peroxidase, slides were incubated overnight at 4°C with rabbit polyclonal IgG antibody directed against ATM protein at 1:2000 dilution (PC116-100UG; ATM (Ab-3); Oncogene Research Products, Calbiochem, USA). The antibodies were detected with the avidin-biotin method using diaminobenzidine as chromogen. The slides were counterstained with Mayer haematoxylin, dehydrated and mounted with DPX. Nuclear staining of normal breast epithelium, myoepithelial cells, and fibroblasts served as an internal positive control and was required for appropriate evaluation. The immunostaining was evaluated in CIS and invasive carcinomas, where evaluation of ATM was performed on hot spot areas, counting at least 500 cells, and was correlated with histopathological parameters, microvessel density, and highest vascularization area. Weak cytoplasmic staining observed in a few cases was not considered for statistical analysis. Since normal epithelium usually had 1-10%

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positive cells, ATM expression was considered reduced (<1%), non-reduced (1-10%), or increased (>10%) in tumors.

b. CD34 Immunostaining

Vascular endothelia were stained with anti-CD34 antibody (Dako, Glostrup, Denmark), with the automatic Tech Mate 500 (Dako), using the EnVision+ (Dako) system. Slides were heated for 10 minutes with citrate Dako ChemMate 10mM buffer in a pressure cooker and incubated for one hour at room temperature with the CD34 antibody at 1:50 dilution. Thirty-minute incubation with the marked polymer was done after inhibition of the endogenous peroxidase, and the slides were then developed with diaminobenzidine, counterstained with Mayer haematoxylin, and finally dehydrated and mounted with DPX.

Image analysis

Microvascularization evaluation by CD34 endothelial expression was performed with an image analysis system (Microimage; Olympus Europe, Hamburg, Germany). The data obtained after this process were the microvessel density/mm² (number of microvessels divided by the size of the field on the screen monitor: 0.05209237 mm²), and the highest microvascularization area (area occupied by microvessels in a given field), considering for the analysis the highest microvessel density value and the largest area among the five evaluated fields.

Statistical analysis

The χ^2 , or Fisher's Test when appropriate, compared qualitative data. Quantitative data were evaluated with the Student's *t*-test. For statistical purposes, cases were grouped as low (B-R I) and higher (B-R II-III) histological grades. Similarly, ATM expression was considered reduced and non-reduced. In order to evaluate the existence of interactions between variables,

the relationship between ATM expression and histological grade, as well as with microvascularization, was studied by means of logistic regression analysis.

Results

Normal breast epithelium showed variable nuclear positivity in 1 to 10% of the luminal cells (Fig. 1). In 38 tumors concomitant intraductal carcinoma was also evaluated. The expression of ATM in CIS was similar to the infiltrating component in 71% of the cases and reduced in 23.7% (Fig. 2). Among infiltrating carcinomas, 18 cases (34.6%) showed non-reduced ATM expression, 5 cases (9.6%) had increased expression (Fig. 3a), and 29 cases (55.8%) showed reduced expression (Figure 4a). The expression of ATM was significantly reduced in higher-grade ductal carcinomas (B-R II-III) when compared to low grade ones (B-R I) (Fisher's Test $p=0.021$) (Table 1). There was no correlation between ATM expression and tumor size, tumor ploidy (ATM expression grouped together as "reduced" (<1%) and as "non reduced and increased"), global S phase (grouped as <4% or >4%), estrogen and progesterone receptor status (grouped as <10% or >10% positive cells), lymph node status, and histological classification. The two mucinous tumors showed non-reduced and increased expression, respectively. The staining in the two medullary type tumors was reduced in one and absolutely negative in the other, whereas the papillary tumor showed reduced expression, and the adenoid cystic carcinoma had non-reduced nuclear

Table 1: ATM expression in infiltrating ductal carcinomas.

GRADE	I	II-III
Reduced ATM	6(23.1%)	20(76.9%)
Non-reduced ATM	10(62.5%)	6(37.5%)

Expression of ATM gene is significantly reduced in higher-grade ductal carcinomas (Fisher's Test, $p=0.021$).

Table 2. Summary of the results of ATM expression and angiogenesis parameters in breast carcinomas according to histological type.

ATM EXPRESSION	MICROVESSEL DENSITY MEAN*	P=	MICROVESSEL AREA MEAN**	P=
All types				
reduced ATM	290,76	0.056	2742,73	0.005
non-reduced ATM	238,75		1894,88	
Ductal				
reduced ATM	291,37	0.077	2948,67	0.001
non-reduced ATM	236,37		1902,47	

*: microvessels/mm² ; **: microns²

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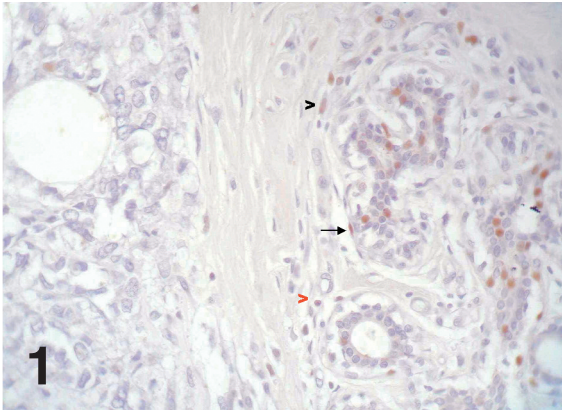


Fig. 1. ATM expression in scattered nuclei of normal ducts. Nuclear positivity in some fibroblasts (black arrowhead), lymphocytes (red arrowhead), and myoepithelial cells (arrow) was also observed. x 40

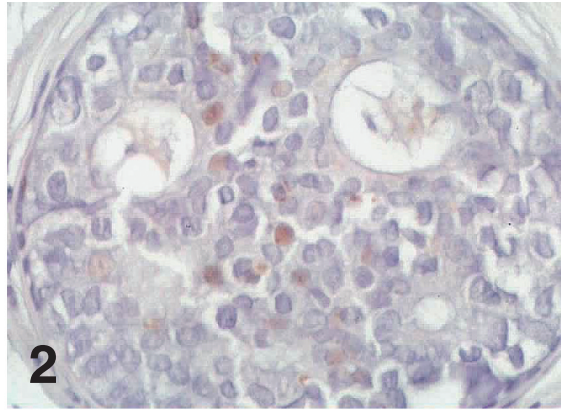


Fig. 2. ATM expression in a focus of in situ carcinoma. x 400

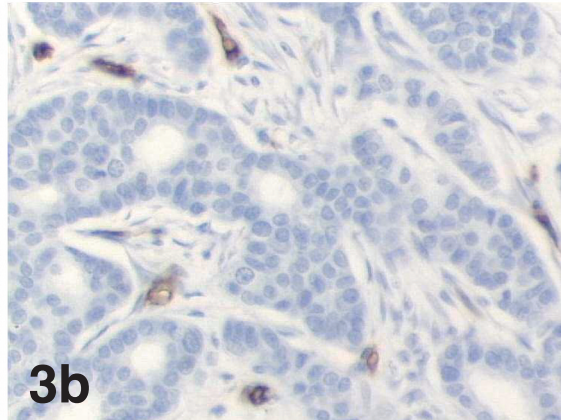
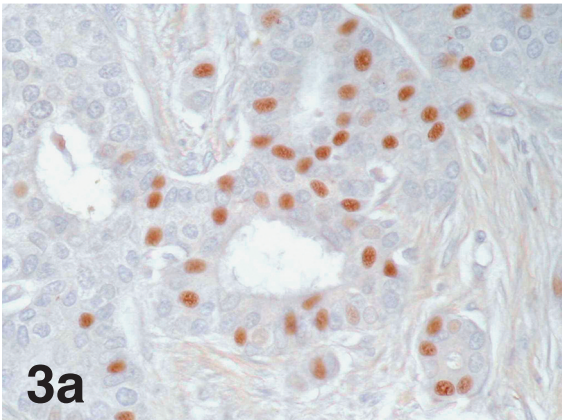


Fig. 3. a. ATM expression in low grade infiltrating carcinoma. x 400. **b.** Same case stained for CD34 with low vascular area and microvessel density. x 400

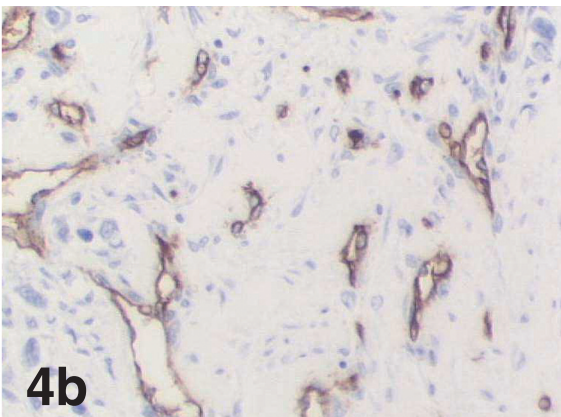
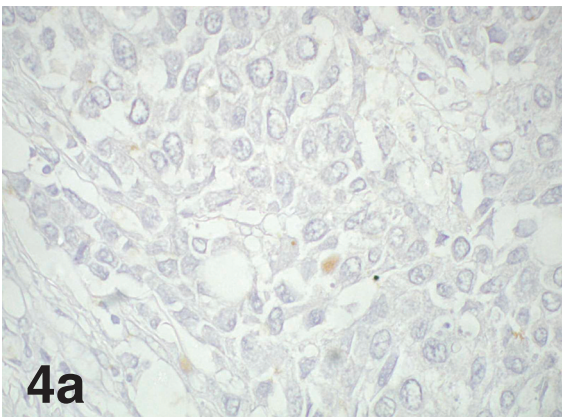


Fig. 4. a. Almost negative ATM expression in a high grade infiltrating ductal carcinoma. x 400. **b.** Same case stained for CD34 showing high vascular area and microvessel density. x 400

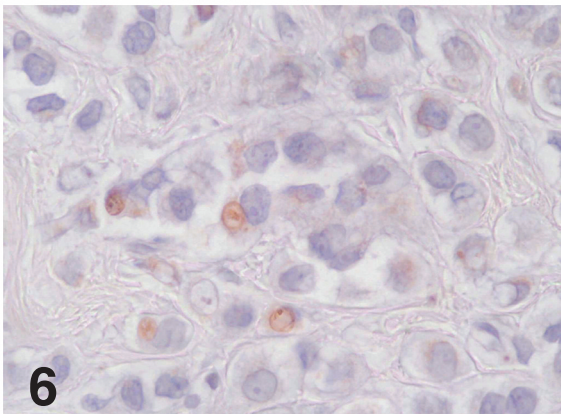
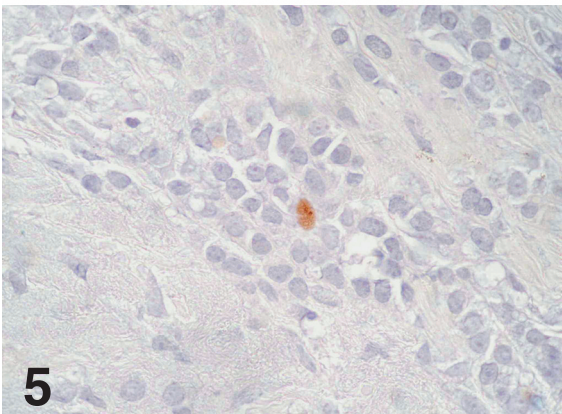


Fig. 5. Reduced ATM nuclear expression in an infiltrating lobular carcinoma. x 400

Fig. 6. ATM nuclear and intravascular positivity in infiltrating lobular carcinoma. x 600

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staining. Three of the four lobular carcinomas had reduced expression (Fig. 5), although this was not statistically significant. Two of them also showed cytoplasmic staining, one within cytoplasmic vesicles (Fig. 6). Some weak cytoplasmic staining was seen in a few other cases (not shown).

There was a significant negative correlation between ATM expression and microvascular area (Student's *t*-test, $p=0.005$), since the mean microvascular area was higher in the cases with decreased ATM of all histological types. This correlation was even higher when only ductal carcinomas were considered ($p=0.001$) (Table 2) (Figs. 3b, 4b). This correlation did not reach statistical significance when ATM expression was compared with the microvessel density in all histological types ($p=0.056$) and in only ductal carcinomas ($p=0.077$).

Since, as mentioned before, ATM expression also correlated with histological grade in ductal carcinomas, regression analysis was used to decide whether or not the ATM/microvessel area relationship was dependent on grade. This test confirmed the independence of such relationship ($p=0.155$).

Discussion

The *ATM* gene is attracting attention as a potentially important target during carcinogenesis given its location at a crossroads of several cell cycle and apoptosis control pathways. ATM is located within a region in chromosome 11q22-23 that frequently undergoes loss of heterozygosity (LOH) in sporadic breast cancer, with a reported 4 to 7-fold increase in breast cancer risk in heterozygous women (Geoffroy-Perez et al., 2001). However, direct mutational analysis has failed to clearly support a role for mutant ATM alleles in breast carcinogenesis (Kovalev et al., 2000), and the role of sequence polymorphisms is also unclear. Also, there is not always a correlation between tumors with LOH and decreased ATM expression according to Scott et al. (2002), but this has been examined in a limited number of cases. Previous studies have shown that ATM mRNA is downregulated in breast cancers when compared with normal tissue by competitive RT-PCR (Waha et al., 1998). The mechanisms of ATM mRNA regulation are not well known, but ATM mutations within the coding region of ATM decrease the mRNA content by abnormal splicing. Decreased ATM levels in carcinomas might also result from non-mutational mechanisms like hypermethylation of ATM gene-promoter regions, involving transcriptional or translational levels with loss of gene function. This is a frequent molecular alteration in human carcinomas, and it has been reported in lung (non-small cell), colon, pancreas, hepatocarcinoma, as well as in breast and human colorectal cell lines, (Kim et al., 2002; Widschwendter and Jones, 2002). Nevertheless, Allinen et al. (2002) found such methylation only in 10% of breast tumors, indicating the important role of other molecular alterations in breast

carcinogenesis.

The existence of other mechanisms of regulation of this protein is supported by the existence of protein overexpression in hyperplasia and in sclerosing adenosis (Clarke et al., 1998; Angele et al., 2004b), two non-neoplastic conditions of breast tissue. Thus, many genomic alterations involving ATM seem to produce a truncated or scarce protein, rendering immunohistochemical analysis an important tool for the evaluation of the functionality of this gene. The results reported here, although from a limited series of cases, show that ATM immunohistochemical expression is downregulated in a significant proportion of breast tumors. They confirm initial and subsequent studies by Angele et al. (2000, 2003a, 2004b), who found frequent reduction in nuclear ATM staining intensity in cases of infiltrating carcinomas, and malignant myoepithelial tumors, as well as those of Kairouz et al. (1999), who showed a reduction or absence of ATM immunoreactivity in more advanced stages of breast carcinoma, with 33% of invasive carcinomas with reduced expression and 71% of metastases. ATM protein is predominantly present in the nucleus, but it is also detected and localized in cytoplasmic and membrane associated vesicles, peroxisomes and endosomes. Kairouz et al. (1999) report strong cytoplasmic expression in normal duct cells in up to 80% of cases. We observed cytoplasmic ATM expression in scattered cells in some tumors. Interestingly, 2 of 4 lobular carcinomas showed such staining; one of them within cytoplasmic vesicles, the meaning of which we cannot infer from our study, but it could be related to ATM cytoplasmic protein transport. In fact, a defective peroxisome and endosome function could be due in part to loss of ATM from these organelles, contributing to oxidative stress and defective exocytosis described in A-T cells, as part of the wide spectrum of signaling defects in A-T (Watters et al., 1997).

We also evaluated ATM expression in concomitant *in situ* lesions, which showed no significant differences with the infiltrating component in most cases. Contrary to previous reports, we carefully evaluated the correlation of ATM expression and histopathological parameters such as differentiation and tumor type. Our results show a significantly reduced ATM expression in high-grade ductal infiltrating carcinomas when compared to non high-grade ones ($p=0.021$), as well as in 3 out of 4 lobular type carcinomas analyzed. These results suggest that abnormalities in the expression of this gene might be more frequent in a subset of breast tumors with special phenotypes related with more aggressive behavior or lobular differentiation. Similarly, Ding et al. (2004) found a high number of abnormalities in double-strand-break checkpoint/repair genes ATM, BRCA1 and TP53, in high-grade tumors, supporting that these genes belong to a common functional pathway whose impairment is associated with breast cancer pathogenesis.

ATM acts on multiple substrates such as p53,

BRCA1, p21/WAF1, CHK1, CHK2 and SMC1, and serves as a link between DNA damage, changes in chromatin structure and downstream signaling events (Gatei et al., 2000; Chun and Gatti, 2004; Lavin et al., 2004; Lukas et al., 2004) with a potential pathogenic function in tumorigenesis. Although ATM abnormalities have been correlated with gross chromosomal alterations given its role in genomic stability control, we found no association between ATM downregulation and aneuploidy or other clinicopathological parameters. This absence of association suggests that impaired ATM expression in breast tumors might not suffice for causing deregulation in parameters such as chromosomal content or certain phenotypic characteristics.

Neoangiogenesis is a key event in tumor development and progression and its evaluation has been proposed as a potential prognostic factor in breast and other types of tumors. Given the existence of aberrant vascular structures in the A-T syndrome, we postulated that ATM protein downregulation in breast tumors might confer some advantage for tumor angiogenesis by still unknown mechanisms. This hypothesis was supported by our results, which detected increased vascular capacity due to a higher microvascular area in tumors with reduced ATM expression, although our immunohistochemical approach is most likely still underdetecting missense alterations. This finding is consistent with the fact that one of the key clinical features of the A-T syndrome is the presence of ectatic (dilated) aberrant vascular structures in different areas of the body. One potential explanation for the relationship between ATM expression and tumor vascularization is the already demonstrated role of wild type p53 in angiogenesis inhibition by different mechanisms, including VEGF downregulation (Ravi et al., 2000; Linderholm et al., 2001; Sherif et al., 2001). Indeed, once the p53 positive regulation by ATM is considered, a downregulation of the latter might be regarded as an indirect mechanism for increased tumor angiogenesis through VEGF stimulation. This interesting, though initial observation provides a substrate for further studies in larger series of histological homogeneous tumors in which different parameters of angiogenesis and its modulators are evaluated. Other potential mechanisms connecting ATM functions with vascular development must be investigated to provide new insights into the complex system of interactions established between the deregulated cell cycle, apoptosis, and neoangiogenesis in human tumors.

In conclusion, our results show that ATM expression is variable, though frequently reduced, among breast carcinomas, and that this mainly occurs in a subset of high-grade carcinomas. Low ATM expression might thus have effects on crucial cell cycle checkpoints, allowing cells that harbor DNA damage to divide and acquire genetic alterations leading to increased tumor grade. Reduced ATM expression is also associated with increased neovascularization in breast cancers, but a cause-effect relationship is yet to be demonstrated.

Taken together, these results indicate that ATM downregulation might be important at different levels and by different mechanisms in mammary carcinogenesis and that it may significantly contribute to the pathogenesis of breast carcinomas.

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