Research Article

AKT1 Inhibits Homologous Recombination by Inducing Cytoplasmic Retention of BRCA1 and RAD51

Isabelle Plo,[1,2] Corentin Laulier,[1,2] Laurent Gauthier,[2] Fabienne Lebrun,[1,2] Fabien Calvo,[3,4,5] and Bernard S. Lopez[1,2]

[1]Unité Mixte de Recherche Commissariat à l'Energie Atomique (CEA)-Centre National de la Recherche Scientifique (CNRS) 217; [2]Institut de Radiobiologie Cellulaire et Moléculaire, CEA, Fontenay-aux-Roses, France and [3]Institut National de la Sante et de la Recherche Medicale; [4]University of Paris 7; and [5]AP-HP, Hôpital Saint Louis, Clinical Investigation Center, Paris, France

Abstract

AKT1 is frequently up-regulated in sporadic breast cancer, whereas *BRCA1* is frequently mutated in familial breast cancer. Because BRCA1 is involved in homologous recombination (HR), we addressed whether AKT1 also has an effect on this process. We showed that AKT1 repressed HR through cytoplasmic retention of BRCA1 and RAD51 proteins, resulting in a BRCA1-deficient–like phenotype. This process does not require direct BRCA1 phosphorylation by AKT1. The cytoplasmic retention of BRCA1 and RAD51 correlated with activated AKT1 in tumor cell lines and in biopsies from sporadic breast cancers. Under nonpathologic conditions, fibroblast growth factor, which activates AKT1 and stimulates proliferation in fibroblasts, impaired excessive HR without fully inhibiting it, promoting genome stability. Our study reveals that the regulation of BRCA1 and RAD51 is altered in a high frequency of sporadic breast cancers and highlights the role of extracellular AKT signaling-dependent regulation of HR and genome stability. [Cancer Res 2008;68(22):9404–12]

Introduction

Homologous recombination (HR), is a fundamental, evolutionarily conserved process involved in the variability/stability balance of the genome. HR is implicated in gene diversification, molecular evolution, chromosome segregation during meiosis, DNA repair, and resumption of stalled replication forks. A very precise regulation of HR is essential to maintain the equilibrium between stability and diversification of the genome. Indeed, uncontrolled excess HR can lead to genetic changes and genome rearrangements (for review, see refs. 1–3), and unresolved HR intermediates can be toxic (4).

On the other hand, deficiencies in HR can result in genetic instability (5–10). Cells expressing a dominant-negative form of RAD51, which poisons HR, produce more tumors when injected into nude mice (5). BRCA1 and BRCA2, two partners of RAD51 involved in HR (11–15), are frequently mutated in hereditary breast and ovarian cancers (16, 17). HR is part of the DNA damage response and most germ line mutations responsible for familial breast cancer involve genes implicated in the DNA damage response (18). However, the large majority of breast cancers are sporadic cases, and it is still not known whether the DNA damage response is also involved in these cases.

The membrane serine/threonine protein kinase Bα (AKT1), which can be regulated by extracellular factors (19, 20), is an oncogene that is either overexpressed or constitutively activated in breast, ovarian, prostate, pancreatic and thyroid tumors (21). Up-regulation of AKT1 has been reported in 38% to 54% of sporadic breast cancers and in 40% of ovarian cancers (21, 22). AKT1 also stimulates tumorigenesis of cells injected into nude mice (21). Interestingly, AKT1 can phosphorylate BRCA1 *in vitro* (23), but the biological significance of this phosphorylation and molecular association has not been addressed. In the current paper, we analyzed the effect of AKT1 on HR.

Materials and Methods

DNA manipulations. All DNA manipulations were performed as described (24). The constructs encoding the human hemagglutinin-tagged AKT [wild-type AKT1 (wtAKT1), membrane-targeted AKT1 (myrAKT1), and kinase-dead [K179A] (kdAKT1)] were kindly provided by Dr. Hemmings (Friedrich Miescher Institute). PCDNA3b, containing HA-BRCA1, was a gift from Dr. Scully (Dana Farber Cancer Institute) and has been previously described (15). PRSVBRCA1 was a gift from Dr. Foray (Institut National de la Sante et de la Recherche Medicale). P-FLAGCMV2-YFPBRCA1 was gift from Dr. Henderson (Westmead Millennium Institute). [T509A]BRCA1, [T509D]BRCA1 and [S615A]BRCA1, [S615D]BRCA1 were generated using the XL-Quickchange site-directed mutagenesis kit (Stratagene). The p*DR-GFP* plasmid and the *I-SceI* expression plasmid were gifts from Dr. M. Jasin (Memorial Sloan-Kettering Cancer Center).

RNA interference. The small interfering RNA (siRNA) sequence used for human BRCA1 was 5′-UCACAGUGUCCUUUAUGUA-3′ and was from Dharmacon. The siRNA sequence for AKT1 was 5′-CAG CCC UGA AGU ACU CUU U-3′ (Eurogentec), and the nonspecific siRNA sequence was 5′-AGGUAGUGUAAUCGCCUUGUUTT-3′. The siRNAs were transfected into cells using Lipofectamine 2000 (Invitrogen).

Cell cultures. The generation of subline, RG37, containing a single chromosomally integrated copy of the recombination reporter plasmid, p*DR-GFP*, was made as described (25). All cell lines were routinely cultured in DMEM, supplemented with 10% FCS, 2 mmol/L glutamine, and 200 units/mL penicillin, in a humidified 37°C incubator with 5% CO_2. The cultures were screened periodically and verified to be free of *Mycoplasma* infection.

Western blot analysis. Cells were washed in PBS and lysed in denaturing, loading dye buffer for 20 min at 4°C. The samples were boiled for 5 min and subjected to Western blot analysis using anti-AKT, anti-[pS^{473}]AKT, and anti–β-actin antibodies. Antibodies were visualized using the enhanced chemiluminescence detection kit (GE Healthcare).

Recombination assays in human RG37 cells. Cells were plated at 2×10^5 per well in six-well plates and transfected after 24 h with various amounts of *I-SceI* using JetPEI reagent. Cells were trypsinized 72 h later,

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Bernard S. Lopez, Unité Mixte de Recherche Centre National de la Recherche Scientifique (CNRS)/Commissariat à l'Energie Atomique (CEA) 217, CEA, Fontenay aux Roses, 92265, France. Phone: 33-01-46-54-88-35; Fax: 33-01-46-54-89-55; E-mail: bernard.lopez@cea.fr.

©2008 American Association for Cancer Research.

doi:10.1158/0008-5472.CAN-08-0861

Downloaded from http://aacrjournals.org/cancerres/article-pdf/68/22/9404/2600077/9404.pdf by guest on 14 July 2024

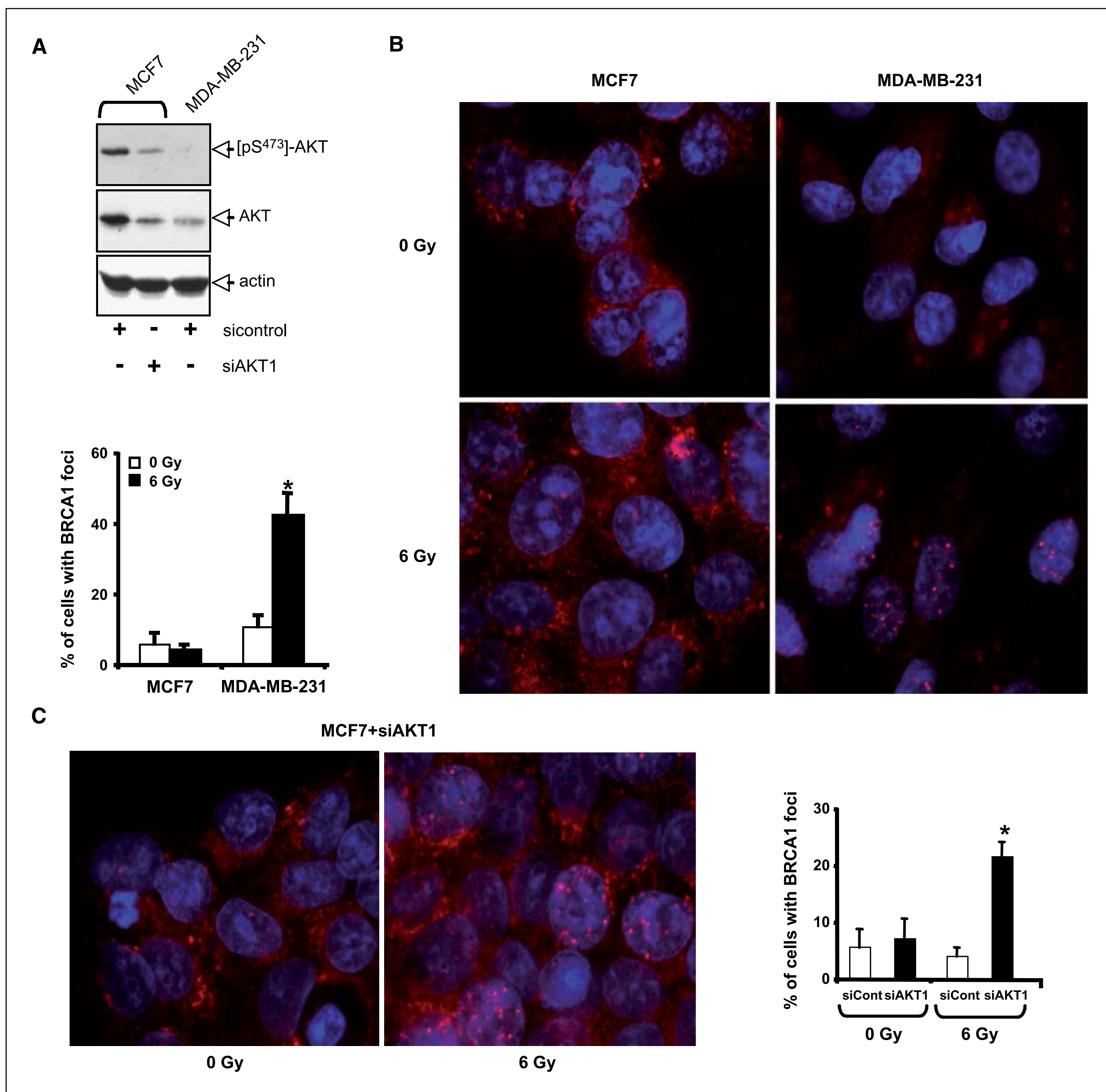


Figure 1. The role of endogenous AKT1 in endogenous BRCA1 foci inhibition in epithelial breast cancer cell lines. *A,* detection of phosphorylated [pS^{473}]AKT1, AKT1, and actin, using specific antibodies, in MCF7 and MDA-MB-231 cell extracts. *B,* IR-induced BRCA1 foci (*bottom*) in MCF7 (*left*) and MDA-MB-231 (*right*) cells. *Left,* histograms are percentages of cells with BRCA1 nuclear foci measured after IR (6 Gy, 12 h). *C,* effect of siRNA (48 h, 120 nmol/L) against AKT1 on BRCA1 nuclear foci induced by IR (12 h) in MCF7 cells. *Right,* histograms are percentages of cells with BRCA1 nuclear foci measured after IR (6 Gy, 12 h). Magnification, 100×.

washed, and fixed in PBS/2% PAF for 15 min at room temperature. GFP^{+} cells were detected by flow cytometry using a FACSCalibur.

Primary breast cancer analysis. All tissues were obtained from the surgery department at Saint Louis Hospital before any treatment was given. None of the patients had a familial history of breast cancer. Biopsies from breast cancer (all ductal invasive carcinoma, stages 2–4, n = 20), normal epithelium (adjacent to tumor, n = 5), and mammoplasty (n = 1) were quick frozen after removal and kept in liquid nitrogen until cryostat sections were cut. The immunohistofluorescence on sections (4 μm) was performed after methanol-acetone fixation and staining with anti-BRCA1 antibodies (1:50, C-20, Santa Cruz or 1:40, SD118, VWR) or anti-RAD51 antibody (1:50, 8121HE, Pharmingen). Ten cryostat sections of each biopsy were used for protein extraction, and Western blot analysis was performed with an anti-[pS^{473}]AKT1 antibody.

Statistical analysis. Results are means ± SEM of at least three independent experiments. Statistical analysis of the cell lines was performed using the Student's t test. Statistical significance (P < 0.05) is indicated by *. For primary breast cancers, comparison of AKT1 levels was done using the Wilcoxon rank sum test with continuity correction, and group comparisons were tested using the Fisher exact test.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/68/22/9404/2600077/9404.pdf by guest on 14 July 2024

Results

AKT1 affects BRCA1 foci formation in epithelial breast cancer cell lines. We first analyzed breast cancer cell lines (MCF7 and MDA-MB-231) spontaneously exhibiting different levels of endogenous AKT1. Western blot analysis showed that AKT1 was highly expressed and constitutively phosphorylated in MCF7 cells. In contrast, AKT1 was expressed and phosphorylated at very low levels in MDA-MB-231 cells (Fig. 1*A*). Interestingly, the formation of endogenous BRCA1 foci, induced by IR, was strongly impaired in MCF7 cells, but not in MDA-MB-231 cells (Fig. 1*B* and Supplementary Fig. S1). Importantly, the silencing of endogenous AKT1 (Fig. 1*A*) restored the formation of IR-induced BRCA1 foci during time (Fig. 1*C* and Supplementary Fig. S1). These data show that high levels of endogenous AKT1 repress the assembly of endogenous BRCA1 foci in MCF-7 breast cancer cells.

Active AKT1 represses BRCA1-stimulated gene conversion. Different mechanisms can take advantage of an intact homologous sequence for double-strand break (DSB) repair: single-strand annealing (SSA), break induce replication (BIR), synthesis-dependent strand annealing (SDSA; ref. 26). Genetic control of these different mechanisms can vary; indeed, whereas gene conversion is RAD51-dependent, SSA is RAD51-independent (27, 28). BRCA1 is a partner of RAD51, which is required in gene conversion (13).

To directly investigate the functional consequences of AKT1 overexpression, specifically on gene conversion, we used a human SV40-immortalized fibroblast cell line, RG37, because it is well characterized for HR (25). Indeed, RG37 cells contain a single chromosomally integrated copy of the *pDR-GFP* substrate, which specifically monitors gene conversion induced by a DSB targeted into the recombination substrate by the meganuclease,

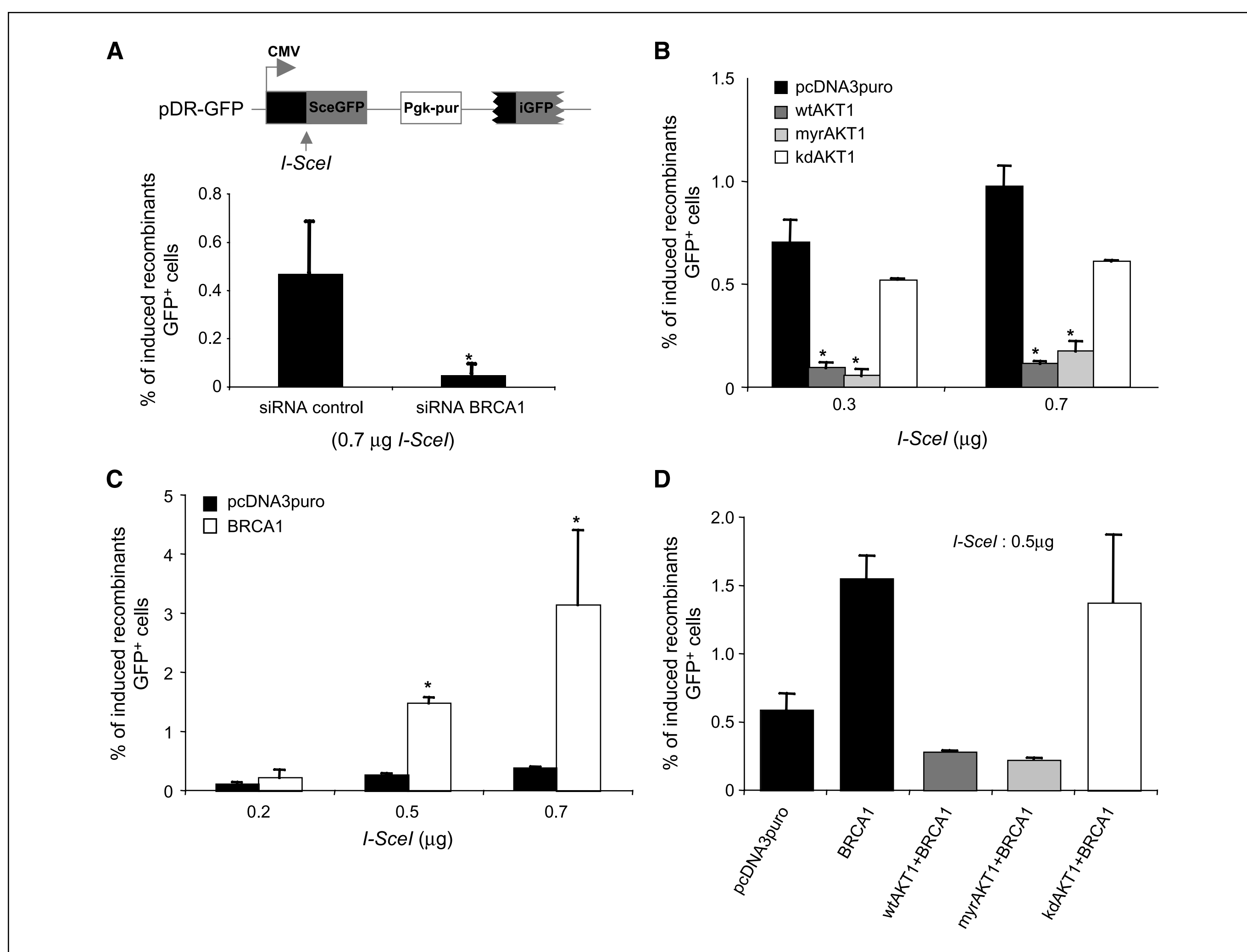


Figure 2. AKT1 inhibits HR in immortalized human fibroblasts. *A,* gene conversion substrate. RG37 immortalized fibroblasts contain one copy of the p*DR-GFP* substrate (25), a tandem repeat of two inactive EGFP genes: SceGFP and iGFP. When the *I-SceI* endonuclease is expressed, a DSB is introduced at the *I-SceI* site in the SceGFP gene. Recombination restores a functional EGFP gene, and the recombinant cells can be monitored by fluorescence detection methods. Importantly, because the iGFP cassette is deleted both in 5′ and in 3′, EGFP fluorescence monitors only gene conversion events not associated with crossing over (29). *Bottom,* silencing of endogenous BRCA1 impaired *I-SceI*–induced HR (histograms). Human RG37 cells were transfected with an siRNA against BRCA1 (120 nmol/L, 24 h) and then transfected with *I-SceI* plasmid (0.7 µg). The percentage of GFP^+ cells was calculated 72 h later. At least three independent experiments were performed. *B,* RG37 cells were cotransfected either with *I-SceI* and empty pcDNA3puro plasmids or with *I-SceI* and different forms of AKT1 plasmids, with a DNA ratio of 1:3. *C,* effect of BRCA1 overexpression on *I-SceI*–induced HR. RG37 cells were either cotransfected with *I-SceI* and empty pcDNA3puro plasmids or with *I-SceI* and BRCA1 expression plasmids, with a DNA ratio of 1:3. At least three independent experiments were performed. *D,* RG37 cells were either cotransfected with *I-SceI* (0.5 µg) and empty pcDNA3puro plasmids or with *I-SceI* and different forms of AKT1 plasmids, in the presence or absence of the BRCA1 expression plasmid, with a DNA ratio of 1:3:3. In all cases, equal total amounts of DNA were used for each cotransfection. At least five independent experiments were performed.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/68/22/9404/2600077/9404.pdf by guest on 14 July 2024

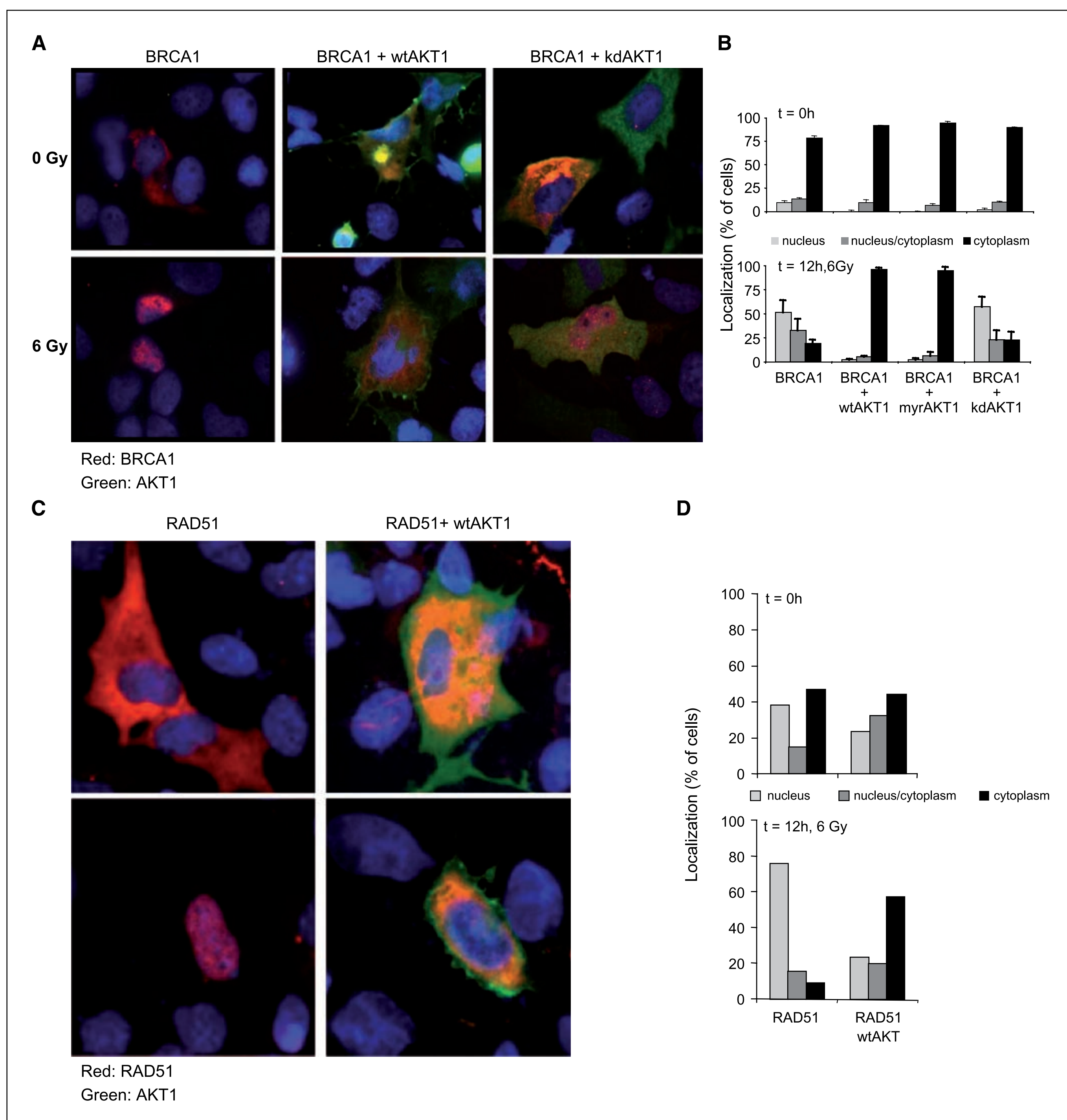


Figure 3. AKT1 affects BRCA1 and RAD51 localization. *A,* RG37 cells were transfected with the BRCA1 expression plasmid alone or cotransfected with different forms of AKT1 expression plasmids and were γ-irradiated. BRCA1 (*red*) and AKT (*green*) localization was visualized by immunofluorescence. *Top lane,* nonirradiated cells; *bottom lane,* irradiated (6 Gy) cells. *B,* percentage of cells showing nuclear, nucleo-cytoplasmic, or cytoplasmic localization of BRCA1 in the presence of different forms of AKT1. *Top,* nonirradiated cells; *bottom,* irradiated cells. *C,* RG37 cells were transfected with the RAD51 expression plasmid alone or cotransfected with AKT1 expression plasmids and were γ-irradiated. RAD51 (*red*) and AKT (*green*) localization was visualized by immunofluorescence. *Top lane,* nonirradiated cells; *bottom lane,* irradiated (6 Gy) cells. *D,* percentage of cells showing nuclear, nucleo-cytoplasmic, or cytoplasmic localization of RAD51 in the presence of AKT1. *Top,* nonirradiated cells; *bottom,* irradiated cells. Magnification, 100×.

I-SceI (Fig. 2*A*; ref. 29). Moreover, this cellular model allows to verify whether AKT1 can also affect BRCA1 functions in different type of cells. We first confirmed that endogenous BRCA1 was functional in HR in these cells, because the silencing of BRCA1 led to a significant reduction in gene conversion (Fig. 2*A*), as expected (13). RG37 cells were then cotransfected with *I-SceI* and different forms of AKT1: wild-type (wtAKT1), a membrane-targeted active form (myrAKT1), or a kinase-dead form (kdAKT1). Coexpression of *I-SceI* and active ectopic wtAKT1 or myrAKT1 led to a 5-fold to a 10-fold decrease in the frequency of *I-SceI*–induced GFP$^+$ cells. The

Downloaded from http://aacrjournals.org/cancerres/article-pdf/68/22/9404/2600077/9404.pdf by guest on 14 July 2024

transfection of kdAKT1 had no significant effect on the frequency of GFP+ cells (Fig. 2*B*). These results show that active AKT1 specifically inhibits gene conversion in human cells.

To more directly assess the role of BRCA1 in HR, we analyzed the effect of BRCA1 overexpression on *I-SceI*–induced HR in RG37 human cells. Cotransfection of *I-SceI* and BRCA1 led to a 6-fold to 8-fold stimulation of gene conversion (Fig. 2*C*). This result is in agreement with a previous work, which shows that overexpression of RAD51, a partner of BRCA1, specifically stimulates gene conversion induced by *I-SceI* (28). This strategy allowed us to focus specifically on BRCA1-controlled HR and, thus, the effect of AKT1 on this specific pathway. We cotransfected BRCA1 and *I-SceI* with different forms of AKT1 and measured the frequency of GFP+ cells (Fig. 2*D*). Both active wtAKT1 and myrAKT1 inhibited BRCA1-stimulated gene conversion. In contrast, kdAKT1 had no effect.

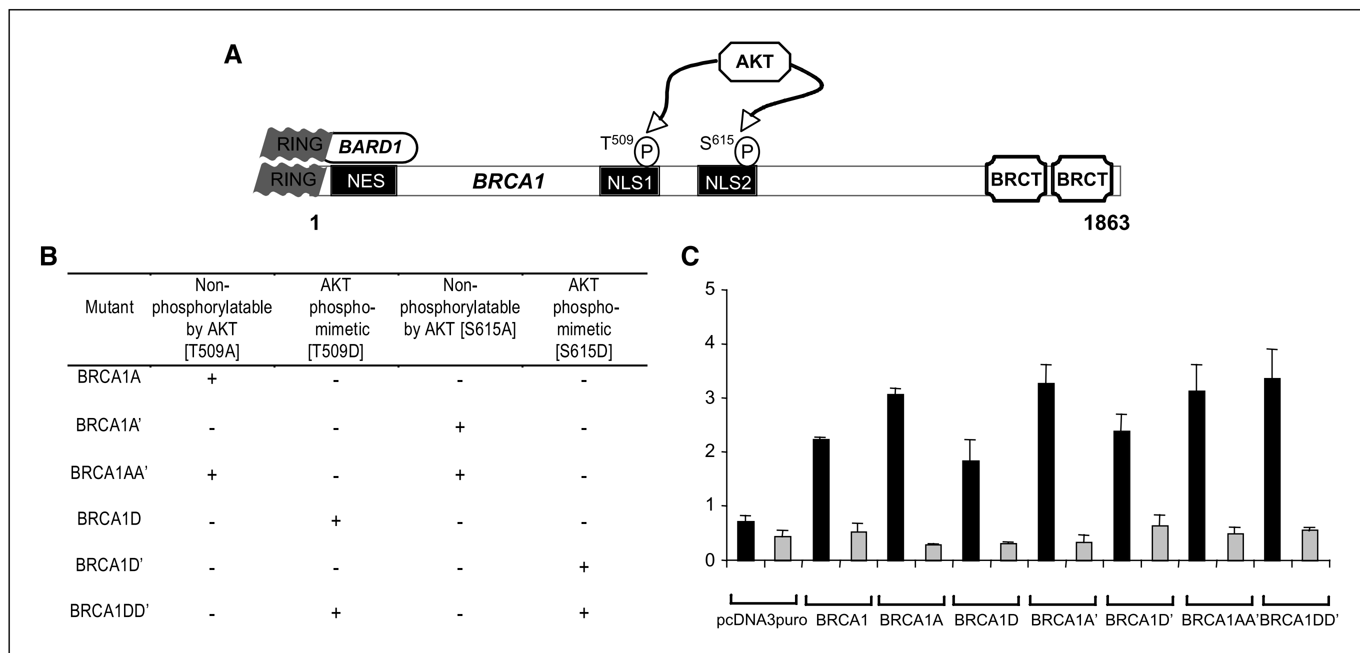
Active AKT1 affects BRCA1 localization. We then investigated the effect of AKT1 overexpression on endogenous BRCA1 foci formation induced by IR in RG37 human SV40 transformed fibroblasts. Coimmunostaining with anti-BRCA1 and anti-AKT1 antibodies showed that, in a given culture, cells expressing AKT1 failed to assemble BRCA1 foci, whereas endogenous BRCA1 assembled into foci in cells lacking AKT1 expression (Supplementary Fig. S2*B*, *left* and *middle* and *C*). These data confirmed the observations made in breast cancer cell lines (see Fig. 1). Expression of kdAKT1 did not inhibit IR-induced BRCA1 foci formation (Supplementary Fig. S2*B*, *right* and *C*). Consistent with the above data, the presence of active wtAKT1 inhibited the formation RAD51 foci after irradiation in RG37 cell lines. The expression of kdAKT1, however, did not induce any change in RAD51 foci formation compared with the parental cells (Supplementary Fig. S2*D*).

The activation of AKT1 often results in the cytoplasmic sequestration of its targets, as shown for CHK1 and BAD (19, 30, 31). Therefore, it is tempting to speculate that BRCA1 is also sequestered in the cytoplasm, upon activation of AKT1. Overexpression of BRCA1 seems to be a valuable tool to unambiguously distinguish between its different subcellular localization. In addition, because AKT1 impairs the up-regulation of HR triggered by the overexpression of BRCA1 (see Fig. 2*D*), it should also affect the subcellular localization of overexpressed BRCA1. In human SV40-transformed fibroblasts, BRCA1 overexpression resulted in its cytoplasmic localization (Fig. 3*A* and *B*). Importantly, two different anti-BRCA1 antibodies, raised against the NH_2 terminus or the COOH terminus, gave similar results. This cytoplasmic localization of BRCA1 seems to be due to the low amount of BARD1 (a partner of BRCA1 essential for its nuclear localization) in human SV40-transformed fibroblasts (data not shown).

Whereas overexpressed BRCA1 was located in the cytoplasm in untreated cells, almost all BRCA1 translocated to the nucleus (Fig. 3*A* and *B*) in few hours after radiation (Supplementary Fig. S3). The presence of active wtAKT1 or myrAKT1 impaired the nuclear localization of BRCA1 after irradiation (Fig. 3*A*, *middle* and *B* and Supplementary Fig. S3*B*), showing that the repression system is robust enough to act on the overexpressed BRCA1. Consistent with the above results, kdAKT1 did not impair IR-induced BRCA1 localization to the nucleus (Fig. 3*A*, *right* and *B*). These data are highly consistent with those obtained in MCF-7 breast cancer cells, in which endogenous BRCA1 and AKT1 are spontaneously highly expressed and endogenous BRCA1 is sequestered in the cytoplasm (see Fig. 1).

RAD51 behaved similarly to BRCA1; its overexpression in human fibroblast resulted in cytoplasmic localization but IR led

Downloaded from http://aacrjournals.org/cancerres/article-pdf/68/22/9404/2600077/9404.pdf by guest on 14 July 2024



Mutant	Non-phosphorylatable by AKT [T509A]	AKT phospho-mimetic [T509D]	Non-phosphorylatable by AKT [S615A]	AKT phospho-mimetic [S615D]
BRCA1A	+	-	-	-
BRCA1A'	-	-	+	-
BRCA1AA'	+	-	+	-
BRCA1D	-	+	-	-
BRCA1D'	-	-	-	+
BRCA1DD'	-	+	-	+

Figure 4. The effect of BRCA1, mutated in AKT1 phosphorylation sites, on gene conversion. BRCA1 can be phosphorylated by AKT1 *in vitro* (23). Using *in silico* analysis (Scansite; ref. 39), we identified another potential AKT1 phosphorylation site in BRCA1 sequence. *A,* diagram represents BRCA1 structures that control its localization. Two potential sites of phosphorylation by AKT1 are present in the two NLS sequences at T^{509} and S^{615}. *B,* list of the different BRCA1 mutants generated. *C,* RG37 cells were either cotransfected with *I-SceI* and empty pcDNA3puro plasmids or with *I-SceI* and AKT1 plasmids in the presence of BRCA1 or BRCA1 mutant plasmids. Gene conversion was analyzed 72 h later by measuring the percentage of GFP+ cells by flow cytometry analysis.

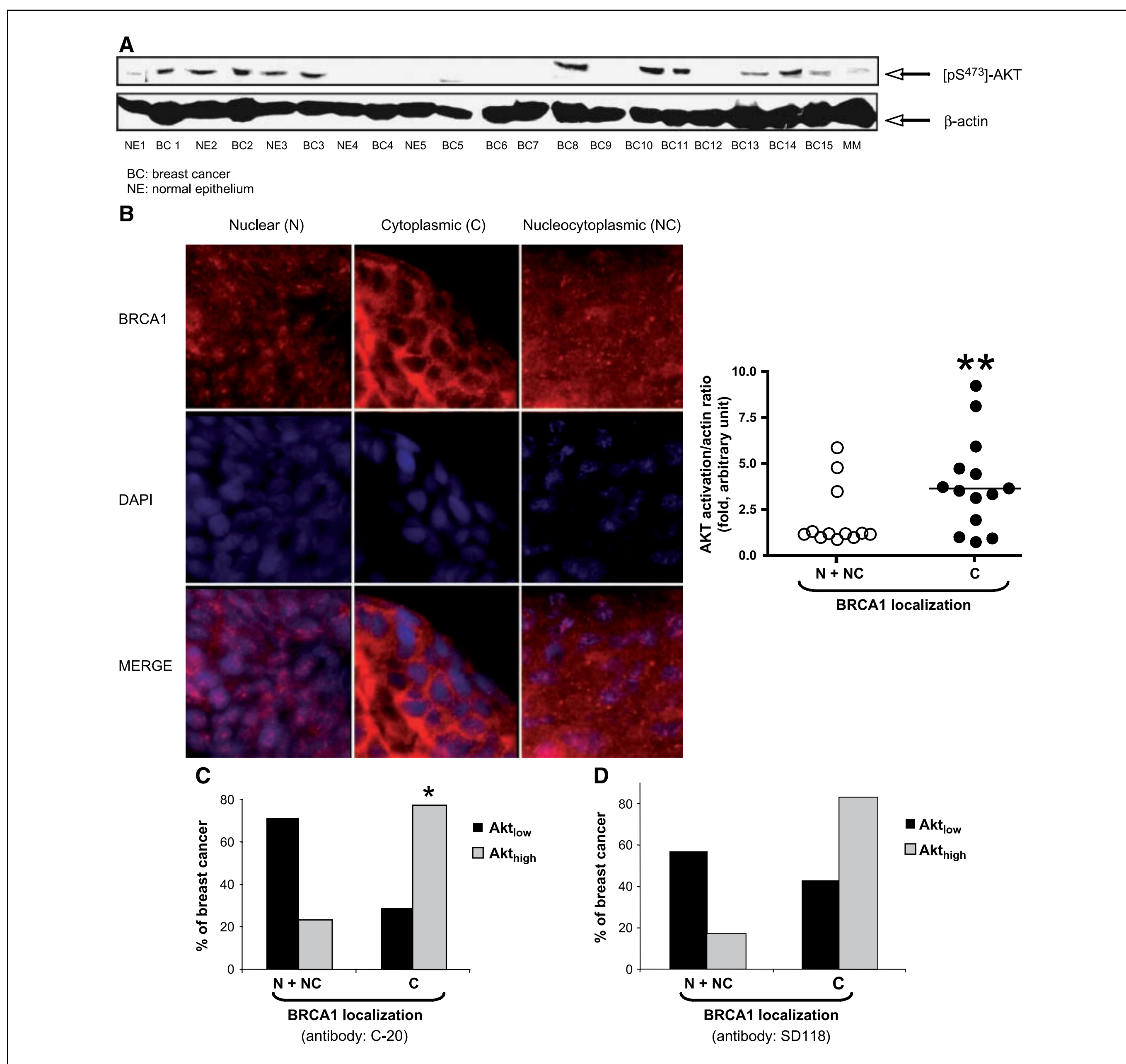


Figure 5. Correlation between AKT1 activation and BRCA1 subcellular localization in sporadic breast tumors. *A,* breast cancer biopsies, normal tissue adjacent to tumors, and reduction mammoplasty tissues were tested for AKT1 activation. Western blot signals were quantified by densitometry, and the specific anti–phosphorylated AKT1/anti-actin signal ratio was used to estimate AKT1 activation. *B,* example of BRCA1 localization in breast cancers, using the C-20 BRCA1 antibody. *Left,* AKT1 activation in tumor cells according to nuclear or nucleocytoplasmic (*NC*) and cytoplasmic staining using anti-BRCA1 antibody (C-20). The cytoplasmic localization of BRCA1 was significantly correlated with the level of activated AKT1 (Wilcoxon test, $P = 0.024$) in breast cancer (*BC*). *C,* we defined two categories: AKT1-low, with AKT1 activation lower than 2-fold, and AKT1-high, with AKT1 activation higher than 2-fold, compared with healthy tissues. Percentages of breast tumors with different BRCA1 localization patterns using the C-20 antibody, according to AKT-low (*black*) or AKT-high (*gray*) activity. *, $P = 0.047$. *D,* BRCA1 localization with SD118 antibody: 83% of AKT-high tissues displayed BRCA1 cytoplasmic localization. The figure shows the percentage of distribution of BRCA1 localization in breast cancer according to AKT-low (*black*) or AKT-high (*gray*) activity using the anti-BRCA1 antibody SD118. *NC,* nucleocytoplasmic; *C,* cytoplasmic. Magnification, 40×.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/68/22/9404/2600077/9404.pdf by guest on 14 July 2024

to the translocation of RAD51 from the cytoplasm to the nucleus (Fig. 3*B* and *C*). The nuclear translocation of RAD51 was also impaired by AKT1 (Fig. 3*C* and *D*).

To assess the effect of AKT1 on BRCA1 nuclear translocation, we transfected an YFP-tagged BRCA1, alone or with wtAKT1, and followed YFP-BRCA1 localization by video microscopy after IR. In the absence of wtAKT1, YFP-BRCA1 translocated from the cytolasm to the nucleus 5 hours after IR. In contrast, in the presence of wtAKT1, most of the YFP-BRCA1 remained trapped in the cytoplasm (Supplementary Fig. S4). These results confirm that active AKT1 impairs the dynamic translocation of BRCA1 to the nucleus.

AKT1 does not affect gene conversion via phosphorylation of BRCA1. Previous studies have shown that AKT1 can phosphorylate BRCA1 *in vitro* (23). Interestingly, the two AKT1 phosphorylation sites are found in the two nuclear localization

signals (NLS) of BRCA1 (Fig. 4*A*). To investigate the role of these two BRCA1 sites in HR, we constructed single or double mutant forms of BRCA1, using site-directed mutagenesis. These mutations resulted in either nonphosphorylatable sites or phosphorylated mimetic sites, mimicking constitutive phosphorylation (Fig. 4*B*). Surprisingly, all of the BRCA1 mutants, including phosphorylated mimetic mutants, efficiently translocated into the nucleus after radiation and stimulated *I-SceI*–induced HR. Moreover, wtAKT1 inhibited nuclear translocation and the stimulatory effects of the BRCA1 mutants, to the same degree, including the nonphosphorylatable mutants (Fig. 4*C* and Supplementary Fig. S5). These results show that the two specific AKT1 phosphorylation sites in BRCA1 are not involved in the repression of gene conversion by AKT1. The situation is, thus, less simple than suggested, and extensive works are required to characterize the molecular mechanisms involved. Nevertheless, the present observations should have very important effects in sporadic breast cancer etiology.

AKT1 activation correlates with BRCA1 and RAD51 cytoplasmic localization in breast cancer. To investigate the implication of our findings in human disease, we analyzed the correlation between AKT1 activation and BRCA1 and RAD51 subcellular localization in sporadic breast cancer. Biopsies of cancer from 20 patients at diagnosis (*BC*) and nontumor specimen [five from normal tissue (*NE*) adjacent to tumors, one from reduction mammoplasty (*MM*)] specimens were analyzed for AKT1 activation by Western blotting (Fig. 5*A*).

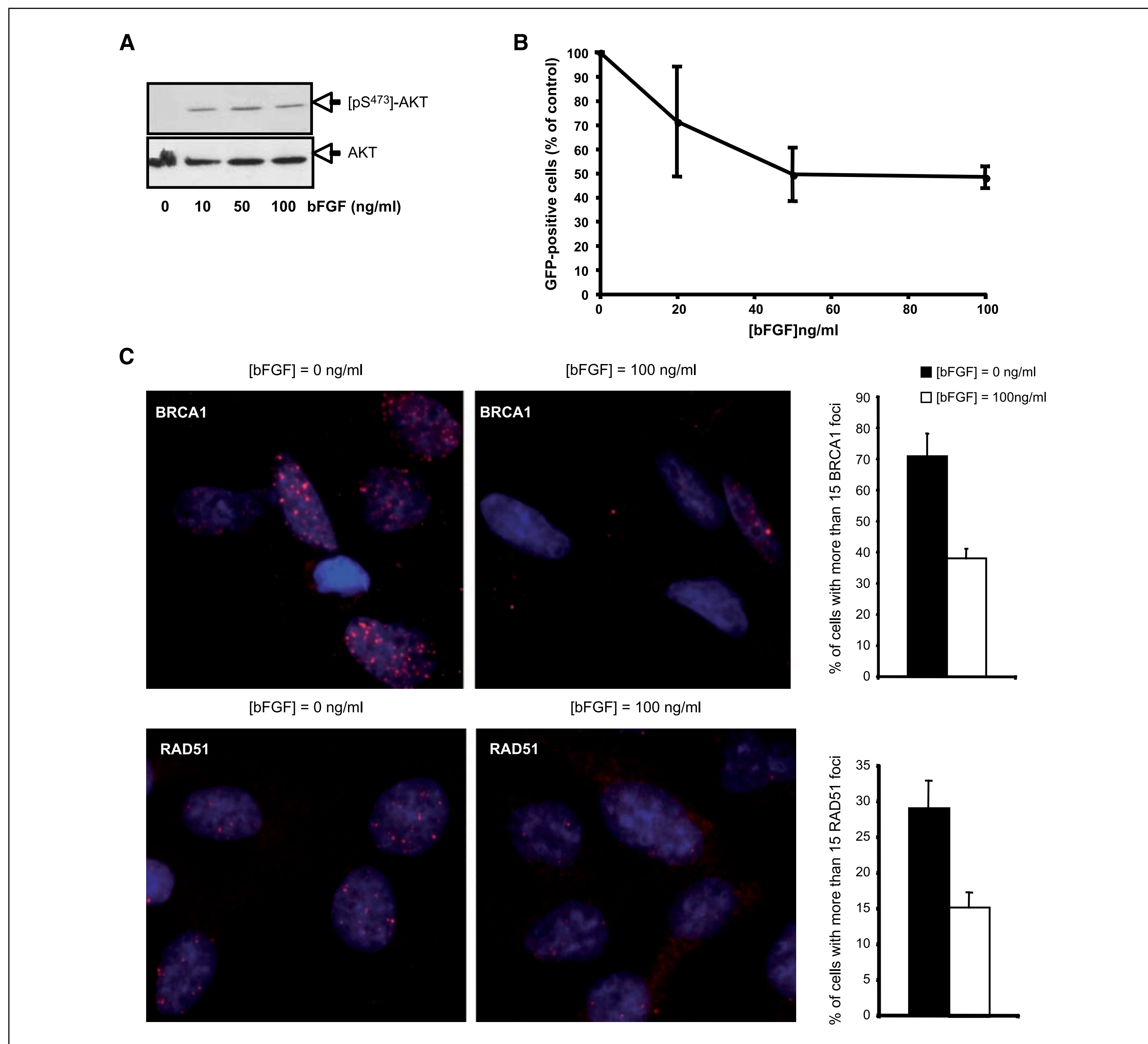


Figure 6. Impact of FGF on HR. *A,* cells were treated with different concentrations of bFGF, and AKT1 expression and [pS^{473}]AKT phosphorylation were detected by Western blot analysis. *B,* cells were transfected with the *I-SceI* expression vector, and different concentrations of bFGF (indicated in the figure) were added to the medium of RG37 human fibroblasts 1 h later. Three independent experiments were performed. *C,* cells were irradiated at 6 Gy for 12 h and radiation-induced BRCA1 (*top*) or RAD51 (*bottom*) foci after treatment with bFGF (*right*) at the indicated concentration for 12 h. The histogram (*left*) corresponds to three independent experiments and shows the frequency of cells with more that 15 foci per cell. Magnification, 100×.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/68/22/9404/2600077/9404.pdf by guest on 14 July 2024

BRCA1 localization was analyzed using two different anti-BRCA1 antibodies. BRCA1 presented three distinct localization patterns (Fig. 5*B*): nuclear, cytoplasmic, or nucleocytoplasmic. The cytoplasmic localization of BRCA1 was significantly correlated with high levels of AKT1 activation in breast cancer (Fig. 5*B*).

We then classified the breast cancer in two groups, AKT1-low (AKT1 activation lower than 2-fold) and AKT-high (AKT1 activation higher than 2-fold, compared with normal epithelium). In AKT-high tumors, BRCA1 displayed a cytoplasmic localization in 77% (with C-20 antibody; Fig. 5*C*) and 83% (with S118 antibody; Fig. 5*D*) of the tumors. Importantly, no exclusively nuclear localization of BRCA1 was detected in any of the AKT-high tumors. In contrast, in AKT-low tumors, BRCA1 displayed a cytoplasmic localization pattern in only 29% of the tumors (Fig. 5*C* and *D*).

Interestingly, RAD51 behaved similarly to BRCA1: 77% of AKT-high tumors exhibited cytoplasmic localization of RAD51 (Supplementary Fig. S6). Immunostaining using the secondary antibody alone as a negative control did not produce any signal, confirming the specificity of the observed localization patterns (Supplementary Fig. S6).

In AKT-low breast cancers, BRCA1 and RAD51 localization mainly show nuclear or nucleocytoplasmic localization patterns. In contrast, AKT1 activation strongly favors the cytoplasmic localization of both BRCA1 and RAD51 proteins. Hence, defective nuclear localization of both BRCA1 and RAD51 is strongly correlated with AKT1 activation in human sporadic breast cancer.

Basic fibroblast growth factor stimulates endogenous AKT1 and reduces HR. AKT1 is an oncogene and its overexpression results in a pathology, as seen in a high frequency of sporadic breast cancers. We have shown that AKT1 overexpression leads to dysregulation of BRCA1, resulting in a BRCA1$^-$like phenotype (without BRCA1 mutation), and a deficiency in HR. Under physiologic conditions, AKT1, which is tightly regulated, is activated in response to extracellular growth factors via the PI-3 kinase to mediate cell proliferation (19). Because HR is essential for cell proliferation, especially for reactivation of blocked replication forks, the repression of HR by AKT1 is an apparent paradox. Two hypotheses can resolve this paradox: (*a*) AKT1 represses HR only under pathologic conditions (constitutive overexpression in cancer) and (*b*) AKT1 impairs excess HR, under physiologic conditions, without completely inhibiting it. Indeed, excess HR can be deleterious to the maintenance of genome stability; uncontrolled HR can lead to loss of heterozygosity, gene inactivation, and profound genome rearrangements (translocation, deletion, amplification). Moreover, unresolved HR intermediates are toxic (1–4).

To address these hypotheses, we treated the human fibroblast cell line, RG37, with basic fibroblast growth factor (bFGF). Activation of endogenous AKT1 was induced by the addition of bFGF to the medium (Fig. 6*A*). HR was decreased, in a dose-dependent manner, by the addition of bFGF to the medium (Fig. 6*B*). Interestingly, at the highest dose, HR was decreased by 2-fold but reached a plateau level and a robust activity was retained. We then tested the effect of bFGF on BRCA1 and RAD51 foci formation after IR (Fig. 6*C*). The addition of bFGF to the medium decreased the amount of BRCA1 foci, as well as of RAD51 foci per cell, in a range similar to the degree of HR repression (compare Fig. 6*B* and *C*). Therefore, as proposed by the second hypothesis, bFGF both activates AKT1 and represses HR but maintains HR at a level sufficient for cell viability. These data suggest that nuclear HR can be very precisely regulated by extracellular factors.

Discussion

Because both excessive and defective HR can lead to genome instability, a very precise regulation of HR is essential to maintain the equilibrium between stability and diversification of the genome. AKT1 may influence HR under normal, physiologic circumstances and during pathologic, chronic overexpression of AKT1. Under physiologic conditions, sufficient HR activity is maintained to ensure genome stability, whereas excessive HR is repressed. In the pathologic situation, HR regulation is out of control, leading to genetic instability and predisposition to cancer.

Physiologic role of HR regulation by AKT1 for genome stability maintenance. Under physiologic conditions, AKT1 is transiently and precisely regulated to avoid uncontrolled cell proliferation; this should lead to subtle, transient, and reversible regulation of HR, as exemplified here in the bFGF experiments. In accordance with our data, serum depletion was shown to strongly stimulate HR (32).

Excessive HR is deleterious for both genome stability and cell viability. Indeed, HR between repeated sequences dispersed through the genome (which are highly frequent) can lead to genome rearrangements (deletions, amplifications, translocations). HR between hetero-alleles or with pseudogenes can lead to gene extinction or loss of heterozygosity (1, 2, 33, 34). Moreover, unprocessed HR intermediates lead to cell toxicity, which can be overcome by inhibiting HR (4). Because the preferred substrate for RAD51 is a ssDNA coated with RPA and because large amounts of this substrate are generated during the S phase, it is essential to precisely control HR during the S phase. This should prevent inappropriate HR initiation in unchallenged cells but should allow HR after DNA damage. A balance between HR initiation and repression is essential; therefore, the control of HR should be precise and, possibly, reversible. Control of HR by extracellular factors should be very adapted to these requirements. We propose that the AKT1 signaling pathway mediates such precise HR regulation.

Pathologic activation of AKT1 strongly inhibits HR and generates genetic instability. Abnormal pathologic situations are encountered in cancer cells (or in cell lines derived in the laboratory). In these extreme situations, AKT1 escapes cellular control and is constitutively activated or overexpressed. AKT1 overstimulation leads to a strong chronic repression of HR, causing genomic instability. Because AKT1 also inhibits cell death, this situation leads to a predisposition to tumors.

Germ line mutations in genes controlling the DNA damage response are responsible for familial breast cancer. However, the majority of breast cancers are sporadic cases. Our findings reveal a novel molecular mechanism underlying AKT1-associated breast cancers. Considering the high frequency of AKT1 up-regulation in sporadic breast and ovarian cancers (30–50% of cases), these findings may be of major importance. Indeed, the present data imply that BRCA1 and RAD51 dysregulation (resulting in a *brca1*-defective phenotype, without requiring BRCA1 mutation) is also important in a high frequency of sporadic breast cancers.

Because AKT1 is up-regulated in 38% to 54% of breast tumors, cytoplasmic localization of BRCA1 should be frequently observed in breast tumor cells. Such frequent cytoplasmic localization of BRCA1 has, indeed, been reported in sporadic breast cancer cells (35). In this study, we found cytoplasmic localization of BRCA1 in 60% of the tumors analyzed, and there was a high correlation between BRCA1 localization and AKT1 activity in primary breast

Downloaded from http://aacrjournals.org/cancerres/article-pdf/68/22/9404/2600077/9404.pdf by guest on 14 July 2024

cancer cells. Moreover, RAD51 behaved similarly to BRCA1, strongly supporting the results obtained with BRCA1. Importantly, the correlation between AKT1 activation and BRCA1 cytoplamic localization was observed using two different antibodies. In addition, RAD51 analysis, one BRCA1 partner, confirmed this aberrant localization, thus, with another antibody against another protein. Finally, these observations are highly consistent with the cellular models, particularly when using YFP-tagged BRCA1, which confirmed cytoplasmic localization, independent of the type of antibody used. Taken together, all the data consistently support the model of cytoplasmic retention of BRCA1 triggered by AKT1 activation.

The present data refute the logical hypothesis that the phosphorylation of BRCA1 by AKT1 at the two NLS sites affects its nuclear shuttling. However, it remains possible that the phosphorylation of BRCA1 by AKT1 might affect other functions of BRCA1, which are not involved in HR. Characterization of the molecular mechanisms involved in BRCA1 retention constitute an exciting challenge for future prospects.

Interestingly, primary breast carcinomas lacking the tumor suppressor, PTEN, show activation of AKT1, cytoplasmic sequestration of the cell cycle checkpoint protein CHK1, accumulation of DSB, and aneuploidy (31, 36). In addition, a nuclear role of PTEN, independent of its role in AKT1 signaling, in controlling RAD51 gene expression and genome stability has also been recently described (37). Finally, it has recently been shown that mutations of PTEN in breast tumor are associated with deficient DSB repair (38). Because BRCA1 is involved in DSB repair, these observations support the data presented here.

In conclusion, our findings shed light, at a molecular level, on an important dimension of the intricate network of cellular processes that connect nuclear, plasma cell membrane, and extracellular pathways in the precise regulation of genome stability. These regulatory processes allow genetic diversity, but protect against genomic instability.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 3/6/2008; revised 6/20/2008; accepted 7/21/2008.

Grant support: La Ligue Nationale Contre le Cancer "Equipes labellisées, La Ligue 2005 and 2008," ANR, INCA, and ARC Fellowship (I. Plo).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. J. Feunteun and his group for helpful discussions, Gaëlle Palierne for technical assistance, all the researchers who provided us with cells and plasmids (see Materials and Methods), and the breast cancer patients.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/68/22/9404/2600077/9404.pdf by guest on 14 July 2024

References

1. Chen JM, Cooper DN, Chuzhanova N, Ferec C, Patrinos GP. Gene conversion: mechanisms, evolution and human disease. Nat Rev Genet 2007;8:762–75.
2. Purandare SM, Patel PI. Recombination hot spots and human disease. Genome Res 1997;7:773–86.
3. Bertrand P, Saintigny Y, Lopez BS. p53′s double life: transactivation-independent repression of homologous recombination. Trends Genet 2004;20:235–43.
4. Gangloff S, Soustelle C, Fabre F. Homologous recombination is responsible for cell death in the absence of the Sgs1 and Srs2 helicases. Nat Genet 2000;25:192–4.
5. Bertrand P, Lambert S, Joubert C, Lopez BS. Overexpression of mammalian Rad51 does not stimulate tumorigenesis while a dominant-negative Rad51 affects centrosome fragmentation, ploidy and stimulates tumorigenesis, in p53-defective CHO cells. Oncogene 2003; 22:7587–92.
6. Deans B, Griffin CS, O'Regan P, Jasin M, Thacker J. Homologous recombination deficiency leads to profound genetic instability in cells derived from Xrcc2-knockout mice. Cancer Res 2003;63:8181–7.
7. Griffin CS, Simpson PJ, Wilson CR, Thacker J. Mammalian recombination-repair genes XRCC2 and XRCC3 promote correct chromosome segregation. Nat Cell Biol 2000;2:757–61.
8. Liu N, Lamerdin JE, Tebbs RS, et al. XRCC2 and XRCC3, new human Rad51-family members, promote chromosome stability and protect against DNA cross-links and other damages. Mol Cell 1998;1:783–93.
9. Sonoda E, Sasaki MS, Buerstedde JM, et al. Rad51-deficient vertebrate cells accumulate chromosomal breaks prior to cell death. EMBO J 1998;17:598–608.
10. Takata M, Sasaki MS, Tachiiri S, et al. Chromosome instability and defective recombinational repair in knockout mutants of the five Rad51 paralogs. Mol Cell Biol 2001;21:2858–66.
11. Marmorstein LY, Ouchi T, Aaronson SA. The BRCA2 gene product functionally interacts with p53 and RAD51. Proc Natl Acad Sci U S A 1998;95:13869–74.
12. Mizuta R, LaSalle JM, Cheng HL, et al. RAB22 and RAB163/mouse BRCA2: proteins that specifically interact with the RAD51 protein. Proc Natl Acad Sci U S A 1997;94:6927–32.
13. Moynahan ME, Chiu JW, Koller BH, Jasin M. Brca1 controls homology-directed DNA repair. Mol Cell 1999;4: 511–8.
14. Moynahan ME, Pierce AJ, Jasin M. BRCA2 is required for homology-directed repair of chromosomal breaks. Mol Cell 2001;7:263–72.
15. Scully R, Plug A, Chen J, et al. Association of BRCA1 with Rad51 in mitotic and meiotic cells. Cell 1997;88:265–75.
16. Martin AM, Blackwood MA, Antin-Ozerkis D, et al. Germline mutations in BRCA1 and BRCA2 in breast-ovarian families from a breast cancer risk evaluation clinic. J Clin Oncol 2001;19:2247–53.
17. Nathanson KL, Wooster R, Weber BL. Breast cancer genetics: what we know and what we need. Nat Med 2001;7:552–6.
18. Walsh T, King MC. Ten genes for inherited breast cancer. Cancer Cell 2007;11:103–5.
19. Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. Genes Dev 1999;13:2905–27.
20. Marino M, Acconcia F, Trentalance A. Biphasic estradiol-induced AKT phosphorylation is modulated by PTEN via MAP kinase in HepG2 cells. Mol Biol Cell 2003;14:2583–91.
21. Sun M, Wang G, Paciga JE, et al. AKT1/PKBα kinase is frequently elevated in human cancers and its constitutive activation is required for oncogenic transformation in NIH3T3 cells. Am J Pathol 2001;159:431–7.
22. Yang H, Wen YY, Zhao R, et al. DNA damage-induced protein 14-3-3σ inhibits protein kinase B/Akt activation and suppresses Akt-activated cancer. Cancer Res 2006; 66:3096–105.
23. Altiok S, Batt D, Altiok N, et al. Heregulin induces phosphorylation of BRCA1 through phosphatidylinositol 3-Kinase/AKT in breast cancer cells. J Biol Chem 1999; 274:32274–8.
24. Ausubel F, Brent R, Kingston R, et al. Current Protocols in Molecular Biology. Boston: John Wiley & Sons, Inc.; 1999.
25. Dumay A, Laulier C, Bertrand P, et al. Bax and Bid, two proapoptotic Bcl-2 family members, inhibit homologous recombination, independently of apoptosis regulation. Oncogene 2006;25:3196–205.
26. Paques F, Haber JE. Multiple pathways of recombination induced by double-strand breaks in *Saccharomyces cerevisiae*. Microbiol Mol Biol Rev 1999;63:349–404.
27. Ivanov EL, Sugawara N, Fishman-Lobell J, Haber JE. Genetic requirements for the single-strand annealing pathway of double-strand break repair in *Saccharomyces cerevisiae*. Genetics 1996;142:693–704.
28. Lambert S, Lopez BS. Characterization of mammalian RAD51 double strand break repair using non lethal dominant negative forms. EMBO J 2000;19:3090–9.
29. Pierce AJ, Johnson RD, Thompson LH, Jasin M. XRCC3 promotes homology-directed repair of DNA damage in mammalian cells. Genes Dev 1999;13:2633–8.
30. Biggs WH III, Meisenhelder J, Hunter T, Cavenee WK, Arden KC. Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. Proc Natl Acad Sci U S A 1999;96:7421–6.
31. Puc J, Keniry M, Li HS, et al. Lack of PTEN sequesters CHK1 and initiates genetic instability. Cancer Cell 2005; 7:193–204.
32. Ludwig DL, Stringer JR. Spontaneous and induced homologous recombination between lacZ chromosomal direct repeats in CV-1 cells. Somat Cell Mol Genet 1994; 20:11–25.
33. Amor M, Parker KL, Globerman H, New MI, White PC. Mutation in the CYP21B gene (Ile-172–Asn) causes steroid 21-hydroxylase deficiency. Proc Natl Acad Sci U S A 1988;85:1600–4.
34. Bertrand P, Rouillard D, Boulet A, Levalois C, Soussi T, Lopez BS. Increase of spontaneous intrachromosomal homologous recombination in mammalian cells expressing a mutant p53 protein. Oncogene 1997;14:1117–22.
35. Chen Y, Chen CF, Riley DJ, et al. Aberrant subcellular localization of BRCA1 in breast cancer. Science 1995; 270:789–91.
36. Puc J, Parsons R. PTEN loss inhibits CHK1 to cause double stranded-DNA breaks in cells. Cell Cycle 2005;4: 927–9.
37. Shen WH, Balajee AS, Wang J, et al. Essential role for nuclear PTEN in maintaining chromosomal integrity. Cell 2007;128:157–70.
38. Saal LH, Gruvberger-Saal SK, Persson C, et al. Recurrent gross mutations of the PTEN tumor suppressor gene in breast cancers with deficient DSB repair. Nat Genet 2008;40:102–7.
39. Obenauer JC, Cantley LC, Yaffe MB. Scansite 2.0: proteome-wide prediction of cell signaling interactions using short sequence motifs. Nucleic Acids Res 2003;31: 3635–41.