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Implication of snoRNA *U50* in human breast cancer

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Abstract

Deletion of chromosome 6q is frequent in breast cancer, and the deletion often involves a region in 6q14-q16. At present, however, the underlying tumor suppressor gene has not been established. Based on a recent study identifying snoRNA *U50* as a candidate for the 6q14-16 tumor suppressor gene in prostate cancer, we investigated whether *U50* is also involved in breast cancer. PCR-based approaches showed that *U50* underwent frequent genomic deletion and transcriptional downregulation in cell lines derived from breast cancer. Mutation screening identified the same 2-bp deletion of *U50* as in prostate cancer in both cell lines and primary tumors from breast cancer, and the deletion was both somatic and in germline. Genotyping of a cohort of breast cancer cases and controls for the mutation demonstrated that, while homozygous genotype of the mutation was rare, its heterozygous genotype occurred more frequently in women with breast cancer. Functionally, re-expression of *U50* resulted in the inhibition of colony formation in breast cancer cell lines. These results suggest that noncoding snoRNA *U50* plays a role in the development and/or progression of breast cancer.

Keywords

snoRNA; *U50*; tumor suppressor gene; breast cancer

Introduction

Breast cancer is a common malignancy and a leading cause of cancer deaths in women (Smith et al., 2007). It results from the accumulation of genetic alterations including activation of oncogenes and inactivation of tumor suppressor genes (Callahan and Campbell, 1989). Identification and characterization of these genes improve our understanding of breast cancer and management of patients. Chromosomal deletion is a hallmark of tumor suppressor genes, because it can reveal recessive mutations, cause haploinsufficiency, or truncate/abolish a gene through loss of heterozygosity (LOH), heterozygous deletion or homozygous deletion (Dong, 2001). Deletion of part or all of chromosome 6, often involving 6q14-q27, is the second most frequent genetic alteration in different types of cancers including breast cancer (Orphanos et al., 1995; Dong, 2001). Deletion at 6q in breast cancer has been detected by different approaches including cytogenetic analysis (Dutrillaux et al., 1990), fluorescence *in situ* hybridization (FISH) (Zhang et al., 1998), comparative genomic hybridization (CGH) (Knuutila et al., 1999), and LOH study (Devilee et al., 1991; Rodriguez et al., 2000). Functionally, introduction of chromosome 6 into tumor cells by micro-cell-mediated chromosome transfer inhibited tumorigenicity in MDA-MB-231 breast cancer cells (Theile et

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al., 1996). These studies suggest that chromosome 6 harbors one or more putative tumor suppressor genes, although the underlying genes have not been finally established.

A recent study identified the snoRNA *U50* gene as a reasonable candidate for the 6q tumor suppressor gene in human prostate cancer (Dong et al., 2008). The *U50* gene, located in the common region of deletion at 6q14.3-15, is transcriptionally downregulated in cancer cells, and has a somatic deletion of 2 basepairs (bps) in cancer cells. Furthermore, deletion of the same 2-bp also occurs in germline, and homozygous genotype of the deletion is significantly associated with clinically significant prostate cancer in a prospectively analyzed cohort of prostate cancer cases and controls (Dong et al., 2008). Functionally, *U50* significantly inhibits *U50*-deficient cancer cell growth.

In this study, we examined whether *U50* is also involved in breast cancer and analyzed its deletion, expression and function in breast cancer cells. By examining germline DNA from both breast cancer patients and control women, we found that heterozygous genotype of the same deletion occurred more frequently in women with breast cancer.

Materials and methods

Cell lines and clinical specimens from breast cancer

Immortalized non-neoplastic breast epithelial cell line BRF-97T and breast cancer cell line BRF-71T1 were purchased from Biological Research Faculty & Facility (BRFF, Ijamsville, MD, USA). All other cell lines were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA) including three immortalized non-neoplastic breast epithelial cell lines (184A1, 184B4 and MCF10A) and 31 breast cancer cell lines (BT-20, BT-474, BT-483, BT-549, CAMA-1, DU4475, HCC38, HCC70, HCC202, HCC1143, HCC1937, HCC1395, HCC1500, HCC1599, HCC1806, HCC2218, Hs 578T, MDA-MB-134, MDA-MB-157, MDA-MB-175, MDA-MB-231, MDA-MB-361, MDA-MB-415, MDA-MB-453, MDA-MB-468, SK-BR-3, SW527, T-47D, UACC893, ZR-75-1 and ZR-75-30). Four peripheral blood cell lines, HCC38BL, HCC1143BL, HCC1937BL and Hs 578Bst, also from ATCC, were obtained from the same breast cancer patients from whom breast cancer cell lines, HCC38, HCC1143, HCC1937 and Hs 578T were established, respectively. Cells were propagated following standard protocols from ATCC. Primary culture of human mammary epithelial cells (HMEC) was purchased from Cambrex (East Rutherford, NJ, USA).

Cancer cells and matched non-cancer cells from 49 clinically primary breast cancer samples were manually dissected following the procedures described in our previous studies (Dong et al., 2006). Genomic DNA for all the samples and RNA for all the cell lines were extracted following standard procedures (Dong et al., 2006). Genomic DNA from a normal human placenta and total RNA from a pool of two normal human mammary tissues were purchased from Clontech (Palo Alto, CA, USA) as controls in specific experiments as stated.

Detection of chromosome deletion at the U50 locus

Chromosome deletion at the *U50* locus was first analyzed by SYBR Green based-real time PCR using the ABI SYBR Green Kit and the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), and then confirmed by duplex PCR using STS marker SHGC-85726 as described previously (Dong et al., 2006). A heterozygous deletion was considered to be present when the ratio of signal intensity for SHGC-85726 marker to that for the control marker in a tumor sample was less than that in the normal human placenta DNA (Clontech). The control marker was from exon 5 of the *KAI1* gene, which is rarely altered at the genomic level in human cancers (Dong et al., 2000).

Expression analysis

Expression levels of *U50* snoRNA in immortalized non-neoplastic breast epithelial and breast cancer cell lines were determined by SYBR Green based-real time PCR using *U50*-specific PCR amplification as described in our previous study (Dong et al., 2008). Briefly, a primer with a linker sequence attached to a *U50*-specific sequence (5′-TCGAGCGGCCGCCCGGGCAGGTATCTCAGAAGCCAGATCCG-3′, linker sequence is underlined) was used to direct cDNA synthesis using the Iscript cDNA Synthesis Kit (Bio-Rad Laboratories, CA, USA). The following pairs of primers, 5′-TCGAGCGGCCGCCCGGGC-3′ (complementary to the linker sequence) and 5′-TATCTGTGATGA TCTTATCCCGAACCTGAAC-3′ for *U50*, and 5′-GTGG TCCAGGGGTCTTACTC-3′ and 5′-TTCAACAGCGAC ACCCACTC-3′ for *GAPDH*, were used to detect *U50* expression. Total RNA from a pool of two normal human mammary tissues was used as normal control. Expression of *U50* in each sample was indicated by the ratio of *U50* expression reading to the reading of *GAPDH*, which was normalized by the normal control.

U50 mutation assay

For the detection of mutation in *U50*, we performed PCR combined with denaturing polyacrylamide gel electrophoresis in all samples, as previously described (Sun et al., 2005). For a shifted band in a sample, which indicated a sequence alteration, genomic DNA from that sample was amplified and PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Germany) and sequenced to reveal the sequence alteration.

Colony formation assay

Expression plasmids for snoRNA *U50* (pSIREN-RetroQ-U50) and vector control (pSIREN-RetroQ), as previously described (Dong et al., 2008), were transfected into breast cancer cell lines MDA-MB-231 and Hs 578T in which *U50* was homozygously mutated using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Briefly, cells were seeded in 12-well plates at a density of 1×10^5 cells per well. On the following day, 1.2 μg of pSIREN-RetroQ-U50 or pSIREN-RetroQ plasmids was transfected in triplicate. Forty hours later, one set of cells were harvested to determine *U50* expression by real time PCR, while another set of cells were grown in selection medium containing 2 μg/mL puromycin for 12 days for colony formation assay. As a positive control, pcDNA3-FLAG-FOXO1A, which expresses known tumor suppressor gene *FOXO1A*, was transfected into MDA-MB-231 and Hs 578T cells in colony formation assay. At the end of selection, cells were fixed with 10% trichloroacetic acid and stained with sulforhodamine B (SRB). Optical densities, which indicated cell numbers, were measured following the protocol in a previous study (Sun et al., 2005).

Association study of U50 mutation in breast cancer

DNA samples from the peripheral blood of 395 women with incident invasive breast carcinomas, collected by Jeff Boyd when he was at the Memorial Sloan-Kettering Cancer Center (MSKCC, USA), were used as cases. All 396 controls were from healthy women with no personal history of cancer, collected in the same institution (MSKCC) and matched for age and ethnicity. Genotyping for *U50* deletion was conducted following the same procedure previously described (Dong et al., 2008). Statistical differences in frequencies of different genotypes between cases and controls were calculated by using the Chi-square test.

Results

U50 is frequently deleted in breast cancer

We first performed real time PCR to determine the deletion status of *U50* in 31 breast cancer cell lines. While no homozygous deletion was detected, 9 of 31 (29%) breast cancer cell lines

showed signal intensities less than half of that in the normal control, suggesting the presence of heterozygous deletions (Fig. 1A). We also used duplex PCR to confirm *U50* deletion in these breast cancer cell lines. Eight of the nine cell lines with heterozygous deletion detected by real time PCR assay also showed heterozygous deletion by this method (Fig. 1B), including BT-483, MDA-MB-175, CAMA-1, HCC202, Hs 578T, HCC1143, BT20 and MDA-MB-231. Deletion frequency of *U50* (8/31, 26%) in the 31 breast cancer cell lines was similar to that reported for the chromosomal locus in previous studies (Noviello et al., 1996;Schwendel et al., 1998;Seute et al., 2001), supporting the candidacy of *U50* as the 6q tumor suppressor gene in breast cancer.

Transcriptional downregulation of snoRNA U50 in breast cancer

The expression of snoRNA *U50* was determined in breast cancer cell lines by real time PCR assay, with normal breast tissues and immortalized non-neoplastic mammary epithelial cell lines as controls. While normal breast tissues had the highest levels of *U50* expression, reduced *U50* expression was detected in four immortalized nonneoplastic mammary epithelial cell lines and all the breast cancer cell lines tested except for HCC1143 (Fig. 2). The reduction was 80% or more for all the cell lines except for HCC1143. Some cell lines had no detectable expression. In HCC1143, the only breast cancer cell line expressing higher levels of *U50*, mutation analysis revealed that *U50* had a homozygous deletion of 2-bp (TT) in the stretch of 4 thymidines (Fig. 3A). All the breast cancer cell lines with heterozygous deletion except for HCC1143 showed a reduction of expression by more than 96%.

Mutations of snoRNA U50 in breast cancer

We first analyzed *U50* mutation in 31 breast cancer cell lines by PCR combined with denaturing polyacrylamide gel electrophoresis and direct sequencing. *U50* showed a homozygous 2-bp (TT) deletion in the stretch of 4 thymidines in three breast cancer cell lines (9.7%, HCC1143, Hs 578T and MDA-MB-231) and heterozygous TT-deletions in one breast cancer cell line (MDA-MB-134) (Fig. 3, A and B). Because the TT-deletion occurred in germline in prostate cancer samples, we also evaluated whether the mutation in breast cancer cell lines was germline or somatic. We analyzed *U50* mutation in cell lines established from peripheral blood cells, HCC38BL, HCC1143BL, HCC1937BL and Hs 578Bst, which were obtained from the same women from whom breast cancer cell lines HCC38, HCC1143, HCC1937 and Hs 578T were derived. Compared to HCC1143 and Hs 578T, which showed homozygous deletion of *U50*, their matched blood cells HCC1143BL and Hs 578Bst showed a heterozygous deletion (Fig. 3A). Taken together with the result that both HCC1143 and Hs 578T showed heterozygous chromosomal deletion at *U50* gene locus (Fig. 1), it is more likely that the wildtype allele of *U50* in these two women was lost through LOH during the development of their breast cancers. Two other lymphocyte lines, HCC38BL and HCC1937BL, showed a wildtype *U50*, the same as their matched breast cancer cell lines HCC38 and HCC1937 (Data not shown). The MDA-MB-231 breast cancer cell line was also homozygous for the deletion but the origin of the mutation could not be determined due to lack of matched normal genomic DNA. LOH could have also given rise to the mutation because it had a heterozygous deletion at *U50* (Fig. 1).

We then examined *U50* mutations in cancer cells and matched non-cancer cells from 49 primary breast cancer samples. Two of the 49 (4.1%) cases showed a homozygous genotype of the TT-deletion in their tumor cells but a heterozygous genotype in their matched normal cells (Fig. 3C), indicating that the TT-deletion occurred somatically in these cases. Another 2 of the 49 cases (4.1%) showed a heterozygous genotype for the TT-deletion in their tumor cells (Fig. 3D), while their matched normal cells showed wildtype genotype, indicating that one of the two *U50* alleles was mutated in these tumors. In 3 of the 49 cases, both cancer cells and matched non-cancer cells showed a heterozygous genotype for the TT-deletion (Data not shown), further indicating that the TT-deletion in *U50* occurs in germline. None of the 49 samples had wildtype

U50 in tumor cells but deletion in normal cells. LOH is common at 6q14.3-15 in breast cancer and, at random, both wildtype allele and the allele with deletion should be lost at an equal frequency. The fact that the loss or somatic mutation only occurred in the wildtype allele but not in the mutant allele in the cases with a germline heterozygous genotype suggests that loss of the wildtype *U50* allele provides a survival advantage for breast cancer cells.

SnoRNA U50 inhibits colony formation in breast cancer cells

To functionally evaluate the candidacy of *U50* as the 6q tumor suppressor gene in breast cancer, we transfected *U50* expression plasmid, along with empty vector control, into breast cancer cell lines MDA-MB-231 and Hs 578T, both of which express reduced levels of homozygously mutated *U50* (Fig. 3A). Colony formation assay was then performed. RNA expression of transfected *U50* was confirmed by real time PCR assay in transfected cells (Fig. 4A). In both cells, ectopic expression of wildtype *U50* significantly reduced colony formation (Fig. 4, B and C). As a positive control for the colony formation assay, transfection of FLAG-pcDNA3-FOXO1A into both cells significantly inhibited colony formation (Fig. 4D).

Association of U50 germline mutation with breast cancer risk

To evaluate whether germline deletion of *U50* is associated with increased risk of breast cancer, as reported in prostate cancer (Dong et al., 2008), we genotyped *U50* deletion in blood DNA samples from 395 patients with breast cancer and 396 samples from control women (Table 1). Of the patients, 2 (0.5%) samples had germline homozygous TT-deletion, 57 (14.4%) had heterozygous deletion, and the rest had wildtype *U50*. In the 396 control samples, 3 (0.8%) had homozygous deletion of TT, 36 (9.1%) had heterozygous deletion, and the rest had wildtype. While the frequencies for homozygous deletion in both cases and controls were rather low and had no significant difference, cases had significantly more heterozygous deletions than controls ($P < 0.05$, Chi-square test).

Both wildtype and mutant alleles of U50 are expressed

Certain samples analyzed, including cell lines MDA-MB-134, HCC1143BL and Hs 578Bst, showed a heterozygous genotype of the *U50* deletion, and RNA could be isolated for expression analysis. We put forward the question whether both wildtype and mutant alleles, or only one of them, is expressed. Using cDNA transcribed from *U50* RNA samples, we amplified *U50* transcripts by PCR and sequenced the PCR products. Both wildtype and mutant *U50* were detected, indicating that both of them are expressed in these samples (Data not shown) and that neither allele has a preference in expression.

Discussion

In this study, we analyzed alteration of *U50* in cell lines and primary tumors from human breast cancer. We found that the *U50* gene underwent frequent copy number loss and transcriptional downregulation in cancer samples. In addition, a 2-bp deletion occurred both somatically and in germline, leading to increased incidence of homozygosity for the deletion in cancer cells. An association analysis showed that heterozygous genotype of the deletion was more frequent in women with breast cancer than those without cancer. Functionally, re-expression of *U50* in breast cancer cell lines Hs 578T and MDA-MB-231 decreased colony formation in culture plates. Consistent with the role of *U50* in prostate cancer (Dong et al., 2008) as well as frequent deletion of chromosome 6q in breast cancer, these results suggest that alteration of *U50* is involved in the development and/or progression of breast cancer.

The existence of multiple tumor suppressor genes at 6q has been suggested by frequent deletion of multiple regions in the long arm of chromosome 6 in breast cancer (Lee et al., 1990; Devilee et al., 1991; Foulkes et al., 1993; Sheng et al., 1996; Mertens et al., 1997; Han et al., 2006) and

by functional suppression of tumor-related phenotype mediated by genes from chromosome 6 (Sandhu et al., 1996; Theile et al., 1996). One region clearly involves 6q14-q16 (Sheng et al., 1996). Several genes have been suggested in several studies as candidate tumor suppressor genes underlying the deletion of 6q, including *SASH1* (Zeller et al., 2003) and *UTRN* (Li et al., 2007) from 6q24, *LATS1* and *ZAC* from 6q24-25 (Varrault et al., 1998; Morinaga et al., 2000; Abdollahi et al., 2003; Basyuk et al., 2005), and *parkin* from 6q25-27 (Cesari et al., 2003). In addition to chromosomal deletion in breast cancer, *ZAC* is ubiquitously expressed in normal human tissues but is downregulated in cancer cells, likely involving promoter methylation (Abdollahi et al., 2003; Basyuk et al., 2005), and inhibits tumor cell growth through induction of apoptotic cell death and G_1 arrest (Varrault et al., 1998). *SASH1* is also frequently downregulated in breast cancers (Zeller et al., 2003). *UTRN* gene expression is downregulated in primary breast cancers, its knockdown induces cellular transformation, and splicing errors and some mutations have been detected in cancer samples (Li et al., 2007). Recently, single nucleotide polymorphisms (SNPs) at 6q22 have been linked to breast cancer (Gold et al., 2008; Rosa-Rosa et al., 2009), although no specific genes have been identified. None of these genes is located in the deletion region at 6q14-16, and no other genes have been established as a tumor suppressor gene for this region, although this region is also frequently deleted in breast cancer (Sheng et al., 1996).

In prostate cancer, germline homozygosity of the TT deletion in *U50* was significantly associated with clinically significant prostate cancer, whereas heterozygous genotype of the deletion was detected at similar frequencies in both cases and controls (Dong et al., 2008). In breast cancer, however, heterozygous genotype of the deletion occurred more frequently in cases than in controls, and germline homozygosity of the deletion was rare in both cases and controls (Table 1). The difference between prostate cancer and breast cancer could suggest that breast cells are more susceptible to *U50* mutation in malignant transformation when compared to prostate cells. As samples sizes in our study are not great, studies with larger cohorts are necessary to clarify the role of germline *U50* mutation in breast cancer development.

SnoRNA is one type of noncoding RNA (ncRNA) in mammals. Although the majority of the human genome encodes for a large number of ncRNA excluding ribosomal RNAs and transfer RNAs (Mattick and Makunin, 2005; Matera et al., 2007), studies are just emerging to indicate roles of ncRNAs in human disease (Mattick, 2004; Kishore and Stamm, 2006). *GAS5*, another snoRNA gene, has also been implicated in breast cancer by its function in arresting the cell cycle and inducing apoptosis and by its downregulation in human breast cancer (Smith and Steitz, 1998; Mourtada-Maarabouni et al., 2009). Our findings further suggest that snoRNA abnormality plays a role in breast cancer development.

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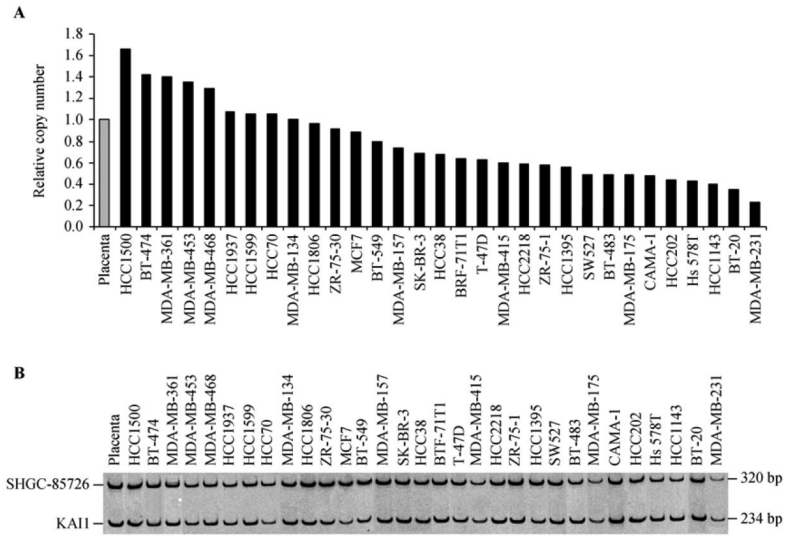

Fig. 1.

Genomic deletion of the *U50* locus in cell lines derived from breast cancer, as detected by real time PCR (**A**) and duplex PCR (**B**). Cell line names are shown below the graph (**A**) or above the image (**B**).

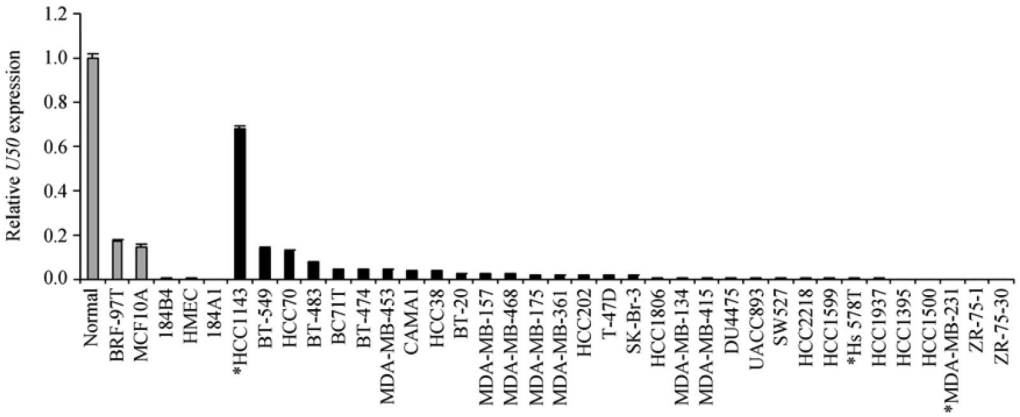

Fig. 2.

Expression of *U50* snoRNA in breast cancer cell lines and control samples detected by real time PCR. Samples with homozygous *U50* TT-deletion are marked by asterisks (*). The 6 samples on the left, marked by gray bars, are from non-cancer cells, while the rest are from cancer cell lines.

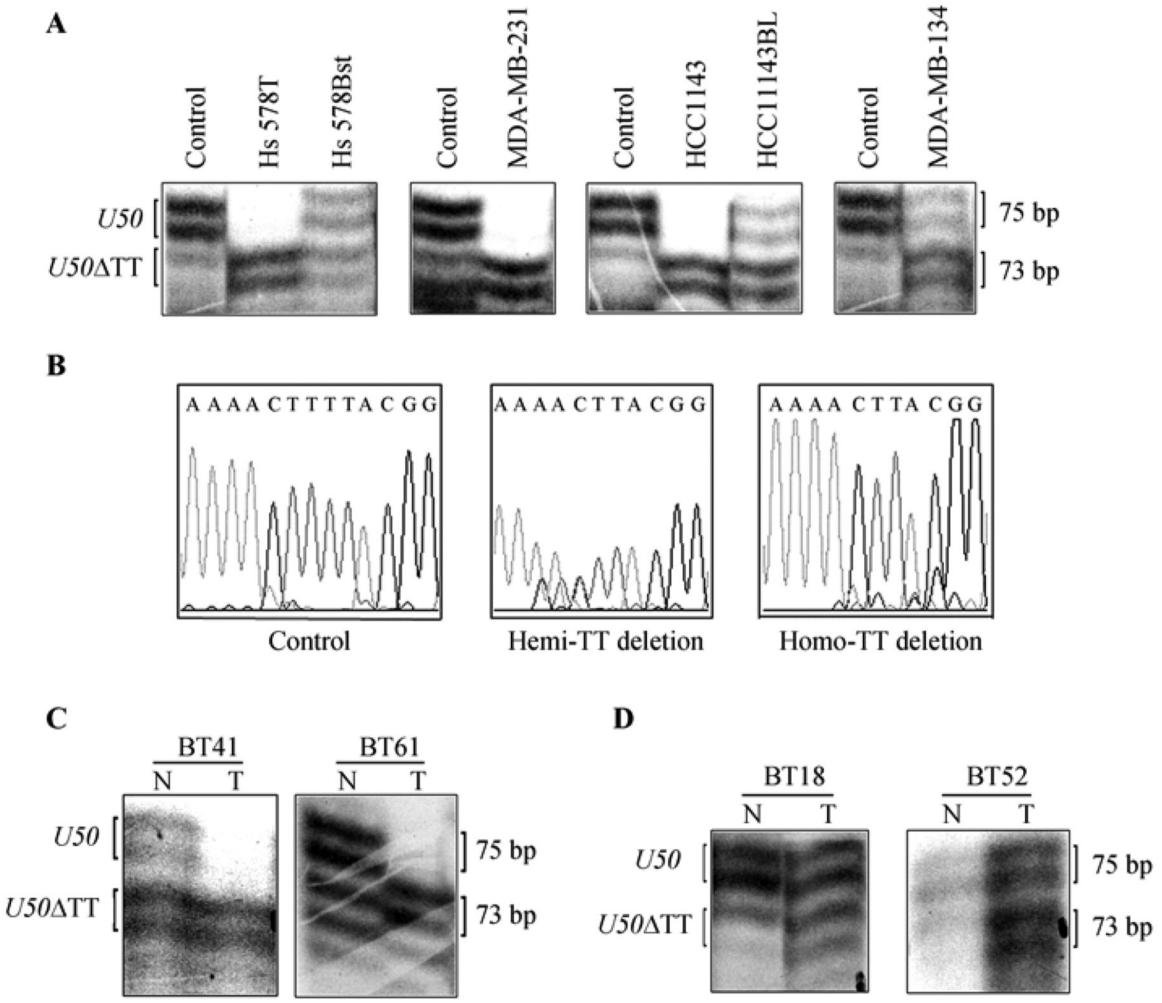

Fig. 3.

Detection of *U50* mutations in breast cancer by gel electrophoresis (**A**, **C** and **D**) and DNA sequencing (**B**). **A:** homozygosity of *U50* mutation in breast cancer cell lines Hs 578T, MDA-MB-231 and HCC1143 and heterozygous deletion in breast cancer cell line MDA-MB-134 and peripheral blood cell lines HCC1143BL and Hs 578Bst. **B:** DNA sequencing results showing wildtype, heterozygous mutant, and homozygous mutant of *U50* in a normal sample and breast cancer cell lines MDA-MB-134 and MDA-MB-231, respectively. **C:** homozygous mutation of *U50* in two primary breast cancer samples compared to their matched normal cells with a heterozygous mutation. **D:** heterozygous mutation of *U50* in two primary breast cancer samples compared to their matched normal cells with wildtype *U50*. In **C** and **D**, T, tumor cells; N, matched non-cancer cells.

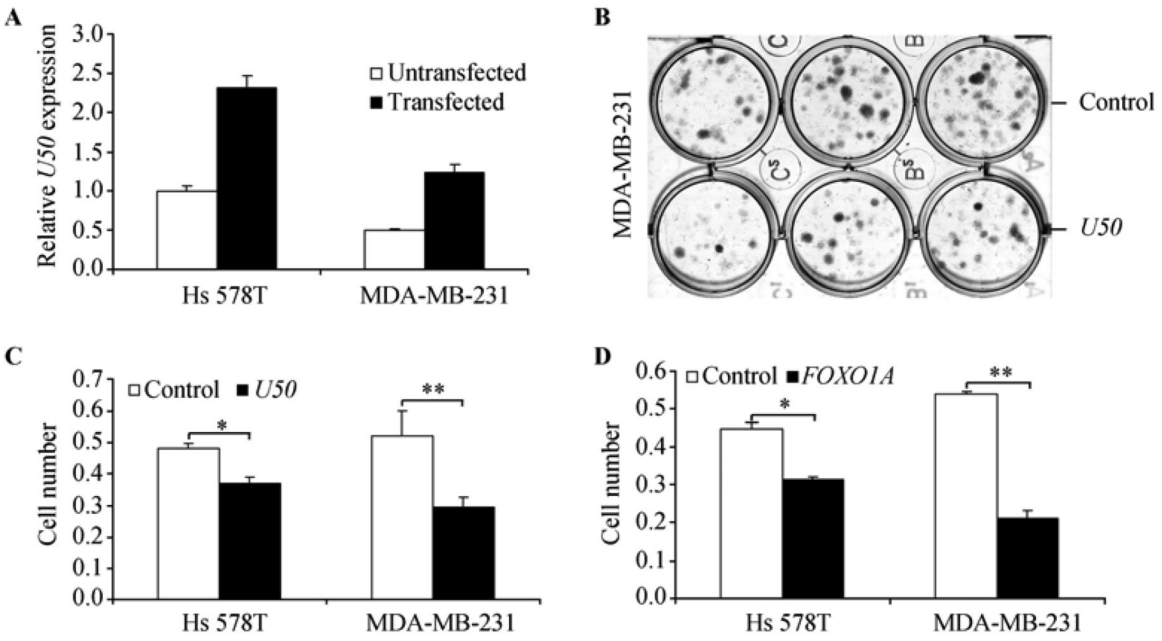

Fig. 4.

Expression of *U50* inhibits cell colony formation in breast cancer cell lines Hs 578T and MDA-MB-231. **A:** confirmation of *U50* expression in transfected cells by real time PCR assay. **B:** an image of tissue culture plate showing cell colonies from MDA-MB-231 cells after transfection with *U50* or control plasmid. **C:** quantitation of cell numbers by measuring optical densities (*y* axis) of cells stained with SRB after *U50* transfection into MDA-MB-231 and Hs 578T cells. *, $P < 0.005$; **, $P < 0.01$. **D:** quantitation of cell numbers after transfection with the *FOXO1A* gene serving as a positive control. *, $P < 0.005$; **, $P < 0.001$.

Table 1

Allelic frequencies of *U50* deletion in a cohort of breast cancer cases and controls

Samples (n)	WT (%) [a]	Het (%) [a]	Hom (%) [a]
Control (396)	357 (90.1)	36 (9.1)	3 (0.8)
Case (395)	336 (85.1)	57 (14.4) [b]	2 (0.5)

[a] WT, wild type; Het, heterozygous genotype; Hom, homozygous genotype.

[b] $P = 0.027$ compared to controls, Chi-square test.