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A somatic mutation in the 5′UTR of BRCA1 gene in sporadic breast cancer causes down-modulation of translation efficiency

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Mutations in the 5′ UTR which cause increment/decrement of translation efficiency have been recently described as a novel molecular mechanism of disease. Alterations in the consensus sequence for the translation initiation may promote context-dependent leaky scanning of ribosomes and/or initiation from a downstream AUG codon. Initiation of translation from a downstream in-frame AUG codon in BRCA1 gene was recently identified in normal cells and possibly in breast cancer. Here we present further insight into BRCA1 translational pathophysiology investigating the role of the canonical structure of the initiation consensus sequence of BRCA1. We have analysed the effect of a somatic point mutation (117 G>C) in position −3 with respect to the AUG of the BRCA1 gene, identified in a highly aggressive sporadic breast cancer. We constructed chimeric genes encoding the luciferase reporter sequence downstream of the wild type or the mutated BRCA1 5′UTR. These transcripts were tested for their activity in *in vitro* and *in vivo* systems. In *in vitro* transcription/translation assays the estimated translation efficiency of the construct with the mutated BRCA1 5′UTR was 30–50% lower than that with the wild type BRCA1 5′UTR. The same chimeric genes were analysed for their expression *in vivo* by transient transfection in human cells. While the two constructs were equally transcribed, the plasmid carrying the mutated sequence produced 70% less luciferase activity compared to the wild type sequence. Finally, to obtain a direct evaluation on translational efficiency *in vivo*, we analysed mRNA translation on translationally active and non-active ribosomes separated from transfected cells. Mutant mRNA was partially localized in subpolysomal particles analytically confirming a polysome recruitment defect. Thus, characterization of BRCA1 5′UTR and translation efficiency seems to provide new insight into BRCA1 role in breast and ovarian cancer pathogenesis. *Oncogene* (2001) **20,** 4596–4600.

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Breast cancer is the most frequently diagnosed cancer in woman from Western countries, occurring in hereditary (5–10%) and sporadic forms. BRCA1 is the gene involved in most cases of breast and ovarian cancer (Hall *et al*., 1990) with mutations thought to be responsible for up to 80% of hereditary breast and ovarian cancers (Szabo and King, 1995). While 9% of sporadic ovarian cancers display a somatic inactivation of BRCA1 (Merajver *et al*., 1995), somatic mutations in this gene have only recently been demonstrated to occur in sporadic breast cancers (Papa *et al*., 1998; Khoo *et al*., 1999). This appears to be in contrast with the hypothesis that BRCA1 is a classical tumor suppressor gene, relevant to both tumor onset and progression (Futreal *et al*., 1994). However, decreased BRCA1 gene expression is frequently found during the transition from carcinoma *in situ* to invasive cancer (Thompson *et al*., 1995). This decrease could be due to factors implicated in complex regulatory mechanisms (Wilson *et al*., 1999) leading to lower levels of BRCA1 mRNA (Magdinier *et al*., 1998; Rio *et al*., 1999; for a review see Wang *et al*., 2000 and Zheng *et al*., 2000).

In a screening for BRCA1 mutations in sporadic breast cancer we have investigated by SSCP analysis and sequencing a population of 96 Italian women with breast cancer and a clinical follow-up of up to 10 years (Papa *et al*., 1998). A somatic mutation in BRCA1 gene was identified in a 32 year-old patient who developed a particularly aggressive breast tumor. We found a single base transversion (G→C) in the second exon of the BRCA1 gene. Analysis of this sequence and that of the BRCA1 pseudogene (GeneBank U77841; Brown *et al*., 1996) did not evidence any similarity, excluding pseudomutation after PCR amplification. This mutation falls close to the end of the 5′ untranslated region (5′UTR), at position −3 with respect to the translation initiation ATG codon, suggesting functional effects on BRCA1 mRNA translation (Iida and Masuda, 1996). In eukaryotes, sequences flanking the AUG codon modulate their

ability to halt the scanning 40S ribosomal subunit and to initiate translation (Kozak, 1999). Mutations that weaken adherence to the 'Kozak consensus motif' (CCPuCCAUGG) in the 5′UTR, negatively affect translation initiation. Particularly relevant for translation initiation efficiency is the presence of a purine at position −3 (Kozak, 1986). This was confirmed by a gene bank study, in which the 5′UTR sequences of about 700 vertebrate mRNAs were compared, demonstrating that 97% of mRNAs have a purine in that position (Kozak, 1987). In order to verify that the G→C mutation at position −3 of BRCA1 mRNA affects mRNA translation, we have compared, by *in vitro* and *in vivo* assays, the translation efficiency of two mRNAs containing the wild type and the mutated forms of the BRCA1 translation initiation sequence, respectively. For *in vitro* analysis we prepared two plasmids encoding, under the T7 RNA promoter, chimeric mRNAs containing a reporter coding sequence (luciferase) preceded by a 19 nt tract encompassing the wt or the mutated BRCA1 translation initiation sequence. As shown in Figure 1, the two constructs, pT7B5Lwt and pT7B5Lmut, encode mRNAs with respectively a 'wt short' and a 'mut short' 5′UTR. To verify if natural BRCA1 mRNA presents the same expression efficiency, a similar construct has also been made with the entire 5′UTR (125 nt) of the wild type BRCA1 mRNA–plasmid pT7B5L long. Expression efficiency of the three constructs has been tested in an *in vitro* transcription/translation assay by measuring the luciferase activity produced in a rabbit reticulocyte lysate supplemented with T7 RNA polymerase. The results of five independent experiments were averaged after normalisation with respect to the value obtained for the construct containing the wt long 5′UTR. Figure 2 (left column of each pair) shows that the two constructs bearing the wt long and the wt short 5′UTRs, respectively, produced the same luciferase activity, allowing the short 5′UTR form to be used in the subsequent experiments. Interestingly, with respect to these constructs, the one with the mutated short 5′UTR led to a significant 30% decrease of luciferase activity production. Antisense transcripts generated by T3 RNA polymerase transcription of the same constructs, used as negative controls, did not produce luciferase activity (not shown). Another series of three experiments was performed in the presence of higher salt concentration, conditions known to support a pattern of context-dependent initiation *in vitro* similar to that seen *in vivo* (Kozak, 1990, 1997). Figure 2 (right column of each pair) shows that, under these conditions, the effect of the mutation at position −3 is even greater (50%). Finally, a reduction in expression was also obtained with the construct containing the mut short 5′UTR compared to the wt short 5′UTR, in an experiment in which uncoupled transcription and translation were carried out in subsequent separate reactions (data not shown). The above results indicate that the G→C mutation at position −3 of BRCA1 mRNA does indeed affect expression efficiency. Since the wild type and the mutant mRNA forms were transcribed from the same T7 promoter, the observed luciferase activity reduction is most likely due to a post-transcriptional effect, reasonably at the translation initiation level.

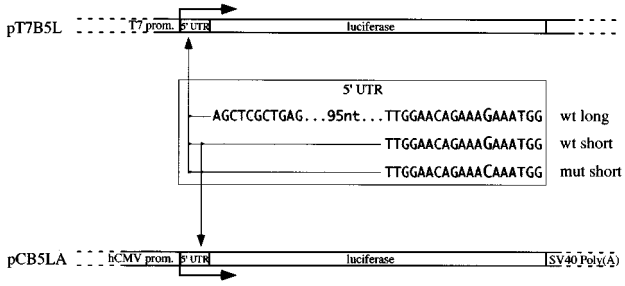

Figure 1 Schematic representation of the constructed chimeric genes. Constructs for *in vitro* transcription/translation experiments (top diagram), were made using the entire luciferase protein coding sequence preceded by the last 15 nt of wild-type (or mutated) 5′UTR of the BRCA1 gene. PCR amplified DNA fragments have been inserted into the pPCR-ScriptAmpSK(+) cloning vector (Stratagene) under the T7 promoter, producing constructs pT7B5Lwt short and pT7B5Lmut short. To generate construct pT7B5Lwt long, containing the luciferase coding sequence preceded by the entire 119 nt wild type 5′UTR of the BRCA1 gene, an RT–PCR was performed on RNA extracted from MCF7 (mammary carcinoma) cell line (Bernard-Gallon *et al*., 1998), with primers designed on the BRCA1 transcript sequence (GenBank accession U14680). Constructs for in *vivo* expression in transfected cells (bottom diagram), containing the luciferase coding sequence preceded by the wild type or mutated 5′UTR of the BRCA1 gene, were obtained by PCR amplification from the above described plasmids pT7B5Lwt short and pT7B5Lmut short. The amplified fragments were inserted under the human CMV promoter in the pUHD cloning vector (kindly provided by Dr Bujard, ZMBH Heidelberg) generating constructs pCB5LAwt short and pCB5LAmut short. All constructs were sequenced

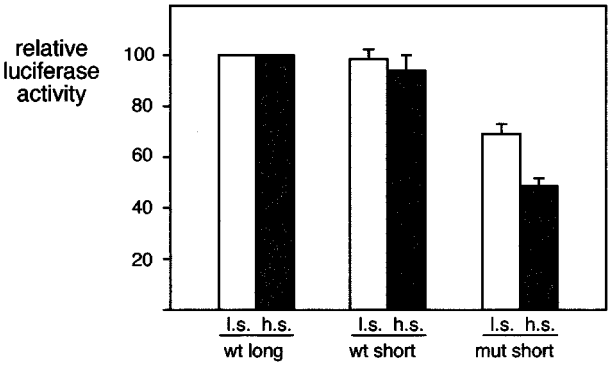

Figure 2 Expression efficiency of 'wt long', 'wt short' and 'mut short' constructs in the *in vitro* transcription/translation assay. 0.5 μg of supercoiled DNA of each construct was incubated in rabbit reticulocyte lysate (Promega) supplemented with T7 polymerase as indicated by the manufacturer (low salt, light grey columns) or at higher salt concentration (+90 mM KAcetate and 2 mM Mg^{2+}, dark grey columns). The results of different experiments (five at low salt and three at high salt concentration) were normalized relative to the value obtained with the wt long construct, and the average values are reported with error bars indicating the standard error of the mean (s.e.m.)

To study the *in vivo* effect of the mutation, constructs were made for transfection and expression in cultured cells. For this purpose the sequences encoding the same above described 'wt short' and 'mut short' chimeric mRNAs, were cloned downstream of the CMV promoter (Figure 1). 0.5 μg of these constructs, pCB5LAwt or pCB5LAmut, were transiently transfected into human HEK293 cells, together with 0.2 μg of pSVβgal which contains the LacZ gene under the control of SV40 promoter (Gorman, 1985) as an internal control for transfection efficiency normalisation. Transfected cells were incubated for 5, 15 and 45 h and then lysed. The results presented in Figure 3, which report the average values of three independent experiments, indicate that, also *in vivo*, the construct with the mutated 5′UTR sequence produced a lower luciferase activity as compared to the wild type: 70 and 50% lower, after 5 and 15 h of transfection, respectively. A difference was still detectable after 45 h of incubation, although it became less evident probably due to saturation of the system.

In order to establish if the observed difference in luciferase activity between cells transfected with wild type or mutant BRCA1 5′UTR is caused by a reduction of translation efficiency of the mutant form, we analysed the levels of chimeric mRNAs in the transfected cells. Total RNA was extracted from the transfected cells and was analysed by Northern blot hybridization with a probe for the luciferase mRNA. No significant differences at the mRNA level have been observed, indicating that transcription efficiency and RNA stability are the same for the two chimeric mRNAs (data not shown).

Finally, to obtain a direct evaluation of the effect of the 5′UTR mutation on translational efficiency *in vivo*, we analysed the transfected cells using a mRNA translation assay (Loreni and Amaldi, 1992). In particular, translationally active ribosomes (polysomes) were separated from non-active (free ribosomal subunits-mRNPs) using a sucrose gradient. For this purpose cytoplasmic extracts were prepared from the transfected cells and analysed. Ten fractions were collected from each gradient while recording the absorbance profile (Figure 4a). The RNA extracted from the gradient fractions was then analysed by Northern blot hybridization using a probe for the luciferase coding sequence. Figure 4b shows that the chimeric mRNA with the wild type 5′UTR is almost completely associated with actively translating polysomes, while the mRNA with the mutated 5′UTR is not completely so, presenting a partial localization in subpolysomal particles that indicates a polysome

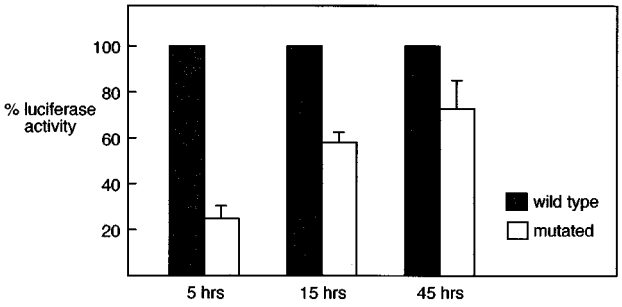

Figure 3 *In vivo* expression efficiency of constructs transcribing luciferase mRNAs with wild type and mutated short forms of the BRCA1 5′UTR. Human embryonic kidney cells (HEK293) were transiently transfected with 0.5 μg of supercoiled DNA (pCB5LAwt or pCB5LAmut), together with 0.2 μg of the control plasmid (pSV2βgal). DNAs were incubated for 20 min with Fugene (Boehringer) in the presence of transactivator, and then added to the cells at a density of 2×10^6 cells/ml. After 5, 15 and 45 h of incubation, cells were rinsed two times with PBS buffer, trypsin treated, recovered and frozen in liquid nitrogen for later analysis. For transfection efficiency normalization, the β-galactosidase activity was measured according to standard procedure (Sambrook *et al*., 1989). The values obtained were used to determine the amount of extract to be used in the luciferase assay. The luciferase enzymatic activity assay was carried out according to the manufacturer's protocol (Promega), and measured by the automated AutoLumat LB 953 Berthold luminometer. The results of three experiments were normalized relative to the corresponding values obtained with the 'wt short' construct, and the average values are reported with error bars indicating the standard error of the mean (s.e.m.)

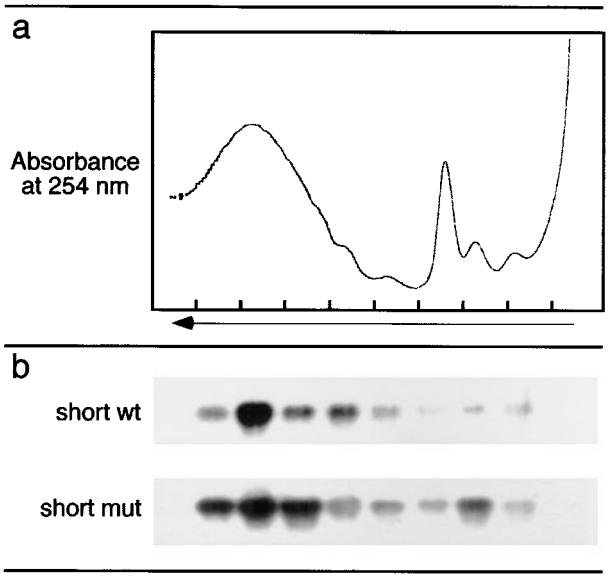

Figure 4 Polysomes/mRNPs distribution, in transfected HEK293 cells, of the two chimeric mRNAs containing respectively the wild type and the mutated 5′UTR. Human HEK293 cell were transiently transfected with 0.5 μg of pCB5LAwt or pCB5LAmut and incubated for 20 h in the presence of transactivator. Cell lysis, sucrose gradient sedimentation of polysomes and analysis of the polysome/mRNP distribution of mRNAs were performed as described (Loreni and Amaldi, 1992). Briefly, cells were lysed directly on the plate with 300 μl of lysis buffer (10 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris-HCl pH 7.5, 1% Triton-X100, 1% NaDeoxycholate, 36 u/ml RNase inhibitor-Pharmacia, 1 mM Dithiothreitol) and transferred to an eppendorf tube. After 5 min of incubation on ice the lysates were centrifuged for 8 min at 10 000 r.p.m. at 4°C. The supernatants were sedimented in 5–70% (w/v) sucrose gradients by centrifugation for 135 min at 37 000 r.p.m. in a Beckman SW41 rotor. Fractions, collected while monitoring the optical density at 254 nm, were extracted and analysed by Northern blot. (a) Absorbance profile of a sucrose gradient showing a typical polysomal pattern. (b) Northern blot hybridization analysis of gradient fractions with probes specific for luciferase coding sequence

recruitment defect. Moreover a minor shift toward lighter polysomes can also be appreciated for the mutated mRNA with respect to the normal one due to a reduced association and/or translation of the mRNA. The results reported here allow us to conclude that the mutated BRCA1 5′UTR with the modified Kozak consensus sequence is defective in promoting translation.

This conclusion is supported by a clinical case described by Morlé *et al*. (1985) which is in line with the importance of the purine in position −3: in an Algerian patient affected by α-thalassemia, the α^{+thal} gene had an A→C change at position −3 relative to the ATG initiation codon, which causes a 30–50% decrease in the translation efficiency of α-globin mRNA (Morlé *et al*., 1986). Similarly, Choong *et al*. (1996) reported a G→A mutation at position +4 (first nucleotide after the ATG) occurring in the androgen receptor (AR) gene in a family with partial androgen insensitivity syndrome (Choong *et al*., 1996). Since the nucleotide at position +4 is an essential element of the Kozak consensus sequence, as is the one at position −3, its mutation results in a defective initiation of translation.

Recently, germ-line or somatic mutations that cause disease through increased or decreased efficiency of mRNA translation have been extensively documented, defining translational pathophysiology as a novel mechanism of human disease (for reviews: Cazzola and Skoda, 2000; Conne *et al*., 2000). The initiation phase of protein synthesis sets the reading frame that is maintained normally throughout all the translation process and regulates the level of initiation and therefore the translation efficiency. Initiation sites in eukaryotic mRNAs are reached via a scanning mechanism which predicts that translation should start at the AUG codon nearest the 5′ end of the mRNA (Kozak, 1999). Great interest has been focused on mechanisms that occasionally allow escape from the first-AUG rule. In fact, mutations in the AUG consensus sequence may shift initiation to other AUG codons which, although not first, are still close to the 5′ end of the mRNA. This shift may alternatively determine a downstream initiation, possibly leading to an N-truncated protein or, if out-of-frame initiation occurs, provide an aberrant protein and/or a premature termination. BRCA1 protein expression can be regulated at the translation level. BRCA1 mRNA presents a second in-frame start codon located 48 nucleotides downstream of the first AUG that may act as an alternative initiation site lacking the first 17 amino acids (Liu *et al*., 2000). The functional significance of ΔBRCA1(17aa) is still unclear but it is possible to speculate different functional effects and/or tissue specific isoforms and pathways (Liu *et al*., 2000). A second mechanism may act on the translational efficiency. As already documented in other diseases (Morlè *et al*., 1985; Cazzola and Skoda, 2000) and experimental designs (Kozak, 1999), transversion at the −3 position of the BRCA1 initiation consensus sequence strongly decreases translation efficiency, allowing almost 30% of the normal synthesis of the encoded protein.

Although the role of BRCA1 in non-familial cancer is still unclear, it is commonly ascertained that reduced expression of BRCA1 protein may play an important role in mammary carcinogenesis, not only in BRCA1-associated familial breast carcinomas, but also in sporadic carcinomas (Jarvis *et al*., 1998; Yoshikawa *et al*., 1999). Reduced BRCA1 expression or incorrect subcellular localization may contribute to the development of high-grade non-familial breast cancer (Wilson *et al*., 1999; Xu *et al*., 1999) and may be associated to the acquisition of distant metastasis. For this reason, BRCA1 expression has been proposed as a novel and useful prognostic marker for this disease type in at least a significant subset of sporadic breast cancer (Seery *et al*., 1999; Lee *et al*., 1999).

Methylation of the BRCA1 promoter has been proposed as one of the mechanisms mediating BRCA1 down-modulation in sporadic breast cancer (Rice *et al*., 1998; Catteau *et al*., 1999; Magdinier *et al*., 1998; Esteller *et al*., 2000). Other epigenetic, as well as genetic, factors may contribute to this effect.

Although patient tissue samples were not available for protein analysis in this study, we have demonstrated that the somatic G→C transversion in the 5′UTR in the second exon of BRCA1 gene is able to affect mRNA translation *in vitro* and *in vivo*, possibly affecting tumor development and/or progression. Further insights in the complex mechanism which regulates BRCA1 synthesis may help identifying new candidate sequences for genetic or epigenetic impairment of BRCA1 availability.

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