Research article

The Icelandic founder mutation BRCA2 999del5: analysis of expression

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Abstract

Introduction: A founder mutation in the BRCA2 gene (BRCA2 999del5) accounts for 7–8% of female breast cancers and for 40% of male breast cancers in Iceland. If expressed, the mutant gene would encode a protein consisting of the first 256 amino acids of the BRCA2 protein. The purpose of this study was to determine whether this mutant protein is produced in heterozygous individuals and, if so, what might be the functional consequences of mutant protein production.

Methods: The presence of BRCA2 999del5 transcripts in fibroblasts from heterozygous individuals was assayed by cDNA synthesis and sequencing. The potential protein-coding portion of BRCA2 999del5 was cloned into the pIND(SP1)/V5-His vector and expressed in COS7 cells. The presence of the mutant protein in cell lysates from heterozygous fibroblasts and from COS7 cells was tested by a number of methods including immunoprecipitation, affinity purification with nickel-coated agarose beads, Western blotting and ELISA, using antibodies to the N-terminal end of BRCA2, antiserum specific for the 16 nonrelevant amino acids at the carboxyl end and antibodies to fusion partners of recombinant proteins.

Results: The frequency of the BRCA2 999del5 transcript in heterozygous fibroblasts was about one-fifth of the wild-type transcript; however, no mutant protein could be detected. Overexpression of BRCA2 999del5 mRNA in COS7 cells failed to produce a mutant protein unless degradation by proteasomes was blocked.

Conclusion: Our results show that the protein product of BRCA2 999del5 is extremely unstable. Therefore, an increase in breast cancer risk in BRCA2 999del5 carriers is due to haploinsufficiency at the BRCA2 locus.

Keywords: BRCA2, BRCA2 999del5, gene expression, haploinsufficiency, Iceland

Introduction

Mutations in BRCA1 and BRCA2 are associated with a high risk of developing cancer of the breast, of the ovaries and of other organs [1]. A large number of cancer-associated mutations in these genes have been described to date; however, few studies have directly probed the functional consequences of individual mutations [2–5]. One question that has thus rarely been addressed is whether the increased risk of cancer is simply due to the lack of one healthy copy of the gene (haploinsufficiency) or whether the gene produces a protein product that interferes with normal cellular processes.

Most nonsense (insertion/deletion) mutants are thought to result in unstable transcripts and in little or no mutant protein production [6]. Exceptions to this rule are found, however, also among the BRCA genes where proteins encoded by the following mutant genes have been detected: BRCA1 5382insC (Breast Cancer Consortium) and BRCA2 6174delT [2]. Perhaps the most intriguing evidence for a potential functional role for truncated BRCA2 mutants comes from the observation that cancer risk is affected by the location of the mutation. Mutations in a central portion of BRCA2, commonly termed the ovarian cancer cluster region, are thus associated with

bp = base pairs; DMEM = Dulbecco’s modified Eagle’s medium; ELISA = enzyme-linked immunosorbent assay; FCS = fetal calf serum; wt = wild type.
significantly higher risk of ovarian cancer but with lower risk of breast cancer than mutations in other regions of BRCA2 [7,8]. This variation in cancer risk is difficult to explain without invoking some effect of a protein product.

A single BRCA2 mutation accounts for 7–8% of breast cancers in Iceland [9]. The Icelandic mutation, a 5 bp deletion in exon 9 starting at nucleotide 999 (BRCA2 999del5), leads to a stop codon at nucleotide 1047 and to premature truncation of protein translation [10,11]. The truncated mutant protein is predicted to have the first 256 amino acids of wild-type (wt) BRCA2, followed by 16 out-of-frame amino acids, and an estimated mass of about 35 kDa. The fact that BRCA2 999del5 represents the only known founder mutation in Iceland has set the stage for extensive population-based genetic and epidemiological studies on the effect of this mutation [9,12,13]. However, to date, no information has been collected on the potential expression of the BRCA2 999del5 gene in heterozygous individuals.

The aim of the present study was to analyze expression of the mutated BRCA2 gene in BRCA2 999del5 heterozygous cells to determine the functional consequences of BRCA2 999del5 at the cellular level.

Materials and methods

Cells and cell lines

Fibroblasts from individuals heterozygous for BRCA2 999del5 were grown in RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 20% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. Overexpressions were performed in COS7 cells (ATCC, Rockville, MD, USA) grown in DMEM (Invitrogen Life Technologies) supplemented with 10% FCS, 100 U/ml penicillin and 100 µg/ml streptomycin.

RNA isolation, cDNA synthesis and cloning of BRCA2 999del5

Total RNA was isolated using the TRIZOL Reagent (Invitrogen Life Technologies). Three micrograms of total RNA were used for cDNA synthesis (First-Strand cDNA Synthesis kit; Amersham Biosciences, Hillerød, Denmark). The open reading frame of BRCA2 999del5 was amplified using a primer pair where the 5′ primer was complementary to the Kozak sequence of the BRCA2 cDNA and the 3′ primer covered a 22 bp region prior to the putative stop codon of the BRCA2 999del5 cDNA. The sequences were GTAAAAATGCCTATTGGATCC for the 5′ cloning primer, and AATGAATTCCTGATGGTTTTTC for the 3′ cloning primer.

The reaction mixture contained 2 µl cDNA template, 2 mM MgCl₂, 15 nmol dNTPs, 15 pmol each primer and 2 U Taq polymerase. The reaction proceeded at 95°C for 5 min, followed by 40 cycles of 94°C for 1 min, 55°C for 45 s and 72°C for 1 min, and finally heating to 72°C for 5 min and cooling to 4°C.

The amplified BRCA2 product was isolated by electrophoresis on a 1% agarose gel, and then the DNA purified (GFX PCR DNA and Gel Band Purification Kit; Amersham Biosciences), cloned into pCR2.1-TOPO vector (Invitrogen Life Technologies) and used to transform TOP10 bacteria. Plasmids were purified from individual bacterial clones (Qiagen Plasmid purification Kit; Qiagen GmbH, Hilden, Germany) and the clones were sequenced (ABI PRIZM Big Dye Terminator Cycle Sequencing Ready Reaction kit; Applied Biosystems, Foster City, CA, USA). After the deletion had been confirmed, the mutated DNA insert was excised from the TOPO-TA plasmid with HindIII and NoII restriction enzymes (New England Biolabs, Beverly, MA, USA) and ligated into the plND(SP1)/V5-His expression vector (Invitrogen Life Technologies).

Transfections and activation of expression

Cells were grown to 60–65% confluency. Four microliters of FuGENE 6 reagent (Roche Applied Science, Hvidovre, Denmark) were added to 250 µl serum-free medium and the mixture was incubated for 5 min at room temperature. After the addition of plasmid DNA (2 µg per transfection), the mixture was incubated for 20 min at room temperature and then added to the cell cultures that had received fresh serum-containing medium. The cell cultures were allowed to recover after transfection for 24 hours, fresh medium was added and the cells were subsequently activated with porasterone A (Invitrogen Life Technologies) at a concentration of 2.5 µg/ml, as recommended by the manufacturer.

Cell lysis, precipitations, and Western blotting

Cells were lysed in RIPA lysis buffer containing 1% Triton X-100, 1% deoxycholic acid, 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 2.5 mM EDTA, 10% glycerol and 0.5 mM phenylmethylsulfonyl fluoride. After lysis, 10% of the lysate was removed (for use as a control) and the rest was incubated with antibody. For immunoprecipitations, 2.5 µl primary monoclonal antibody or 5 µl primary polyclonal antibody were used per precipitation. After the addition of antibody, the samples were subjected to gentle end-over-end shaking for 1–2 hours at 4°C. Subsequently, 50 µl Protein G Sepharose beads (Amersham Biosciences) were added to each sample and the mixture incubated for a further 1 hour at 4°C. The beads were washed with lysis buffer, the supernate was removed and 50 µl of 2 × sample buffer containing β-mercaptoethanol were added to the beads. The samples were boiled for 5 min and were centrifuged for 30 s.

For precipitation of the overexpressed 6×His-tagged proteins, Ni-NTA Magnetic Agarose Beads (Qiagen) were added to the beads. The samples were boiled for 5 min and were centrifuged for 30 s.

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GmbH) were used. For purification of the 6×His-tagged proteins under native conditions, cells transiently transfected with pIND-BRCA2 999del5 or with pIND-LacZ were lysed in lysis buffer containing 50 mM NaH2PO4 (pH 8), 300 mM NaCl, 10 mM imidazole, 1% Tween 20. Ten microliters of 5% Ni-NTA Magnetic Agarose Bead suspension were added to the lysate and incubated for 1–2 hours at 4°C. The supernate was separated from the beads using a Qiagen 12-Tube Magnet (Qiagen) and the beads were washed several times with washing buffer containing 50 mM NaH2PO4 (pH 8), 300 mM NaCl, 20 mM imidazole, 0.05% Tween 20. The proteins were eluted from the beads with 25 µl elution buffer containing 50 mM NaH2PO4 (pH 8), 300 mM NaCl, 250 mM imidazole, 0.05% Tween 20, an equal volume of 2 x sample buffer supplemented with β-mercaptoethanol was added to the eluates, and the samples were boiled for 5 min and centrifuged for 30 s.

Purification of the 6×His-tagged proteins under denaturing conditions proceeded in exactly the same way except that the cells were lysed in a buffer containing 8 M urea, 0.1 M NaH2PO4, 0.01 M Tris–Cl (pH 6.3), and that a buffer containing 8 M urea, 0.1 M NaH2PO4, 0.01 M Tris–Cl (pH 8) was used for elution of the proteins from the beads.

The precipitates and the lysates were separated by electrophoresis on gradient 6–18% polyacrylamide gels and blotted onto a Hybond-P membrane (Amersham Biosciences). The primary antibodies used in this study were: SC1 and SC3 rat monoclonal antibodies raised against the N-terminal and C-terminal sequences of BRCA2, respectively (a generous gift from D Bertwistle and A Ashworth); anti-V5 mouse monoclonal antibody (Invitrogen Life Technologies); anti-actin mouse phosphatase-conjugated antibody (DAKOCytomation, Glostrup, Denmark) diluted 1:3000 in PBS–0.05% Tween 20. The antibody against the putative out-of-frame residues of BRCA2 999del5 was used to detect the presence of the mutated BRCA2 protein. Preimmune serum was used as a control. The secondary antibody used was goat anti-rabbit alkaline phosphatase-conjugated antibody (BAKOCytomation, Glostrup, Denmark) diluted 1:10000 in PBS–0.05% Tween 20.

Northern blotting
Total RNA samples (about 15 µg each) were separated by electrophoresis on a 1% agarose gel containing 2.2 M formaldehyde and 0.02 M 3-((N-morpholino)propane-sulfonic acid, stained with ethidium bromide and visualized by UV. The gels were blotted onto Hybond-N+ membrane (Amersham Biosciences) and RNA was subsequently fixed by exposing the membrane to UV light for 5 min. The BRCA2 999del5 insert of the pIND-BRCA2 999del5 plasmid was excised from the vector with HindIII and NotI. Then 100 ng insert were labeled using the ECL direct nucleic acid labeling system (Amersham Biosciences) and were used as a probe. The labeled probe was added to a hybridization buffer containing 0.5 M NaCl and 5% blocking agent, and the mixture was incubated with the membrane overnight at 42°C. The images were developed using the ECL detection system (Amersham Biosciences).

ELISA
F96 Maxisorp immunoplates from Nunc (Roskilde, Denmark) were coated with cell lysates. The synthetic 16 amino acid peptide alone (the predicted 16 out-of-frame amino acid stretch of BRCA2 999del5), and the same peptide conjugated to keyhole limpet hemocyanin carrier protein were used as controls. Briefly, wells were coated with different dilutions (1:10, 1:100, 1:1000) of lysates prepared from the heterozygous fibroblasts on one hand, and from homozygous wt fibroblasts on the other hand, all made in duplicate. About 250 ng synthetic peptides were used to coat each control well. All the samples were diluted in coating buffer containing 0.15 M Na2CO3, 0.035 M NaHCO3, and 0.003 M NaN3 (pH 9.6), which was also used as a blank. The same experiment was performed with the lysates of the COS7 cells transfected with pIND-BRCA2 999del5 or with pIND-LacZ as control. The antibody against the putative 16 out-of-frame amino acid stretch of BRCA2 999del5 (dilution 1:2500) was used to detect the presence of the mutated BRCA2 protein. Preimmune serum was used as a control. The secondary antibody used was goat anti-rabbit alkaline phosphatase-conjugated antibody (DakoCytomation, Glostrup, Denmark) diluted 1:3000 in PBS–0.05% Tween 20.

Proteasome inhibition
To investigate whether the proteasome plays a role in the instability of BRCA2 999del5 protein, the proteasome inhibitor lactacystin was used to treat cells overexpressing pIND-BRCA2 999del5 or pIND-LacZ. Briefly, 24 hours after transfection, the cells were treated with ponasterone A and consequently with lactacystin at a concentration of 10 µM for different periods of time. The cells were thereafter lysed and subjected to electrophoresis on gradient 6–18% polyacrylamide gel, transferred onto Hybond-P membrane and blotted with α-V5 or with the antibody against the nonrelevant residues of BRCA2 999del5 or SC1 antibody. Blotting with α-actin was performed as a control for protein loading, and blotting with α-p53 was performed as a control for proteasome inhibition.
Results

Expression of BRCA2 999del5 mRNA in heterozygous cells

We tested whether mRNA encoding the mutated copy of the BRCA2 gene was present in cells heterozygous for BRCA2 999del5. cDNA was prepared from heterozygous fibroblasts, and an 822 bp region, covering the open reading frame of BRCA2 999del5, was amplified and cloned into TOPO plasmid. Sequencing of individual cDNA clones confirmed the existence of the mutant transcript. Out of 24 randomly chosen clones, five (approximately 20%) had the 5 bp deletion. Thus, although the mutant gene is transcribed, it is probable that the resulting mRNA is less stable than the mRNA transcript of the wt gene.

No BRCA2 999del5 mutant protein could be detected in heterozygous cells

The existence of mutated protein in fibroblasts heterozygous for BRCA2 999del5 was investigated by Western blotting. Two different antibodies were used for detection of the mutated protein: rat monoclonal antibody raised against amino acids 5–19 of human BRCA2 (SC1), and rabbit antiserum raised against the predicted 16 out-of-frame amino acid stretch of BRCA2 999del5. Rat monoclonal antibody raised against amino acids 3386–3400 of human BRCA2 (SC3) and the SC1 antibody were used to detect wt BRCA2. There was no difference in the banding pattern of lysates of heterozygous samples compared with lysates with wt BRCA2 for any of these antibodies (data not shown). Immunoprecipitations with the SC1 antibody also failed to detect any mutant protein.

Overexpression of BRCA2 999del5 in COS7 cells

The inability to detect mutant proteins in BRCA2 999del5 heterozygous cells by Western blotting could be due to the lack of sensitivity of the antibody reagents. To test this possibility, the open reading frame of BRCA2 999del5 was cloned into the pIND(SP1)/V5-His vector. COS7 cells were transfected with pIND-BRCA2 999del5 and pVgRXR (Ecdysone-receptor plasmid), and 24 hours later expression of pIND-BRCA2 999del5 was induced by ponasterone A. Parallel transfections with pIND-LacZ plasmid were used as positive control. Northern blot analysis showed that BRCA2 999del5 RNA was expressed at high levels in transfected cells (Fig. 1). As the pIND plasmid contains sequences encoding a V5 epitope and a 6xHis tag (poly-His), the cell lysates were probed for recombinant protein by affinity purification with either anti-V5 antibody or Ni-NTA magnetic agarose beads. No overexpressed BRCA2 999del5 protein could be detected either in total cell lysates (Fig. 2a) or after purification on nickel-coated beads or precipitation with α-V5 (Figs 2b and 2c, respectively). Thus, despite efficient transcription of the plasmids and clear detection of the LacZ protein, no mutant BRCA2 protein could be found.

ELISA fails to detect both endogenous and overexpressed mutant protein

BRCA2 has proven to be a poor antigen for antibody production, hampering studies on the functional aspects of the protein. To try to gain a better handle on the BRCA2 999del5 protein product, a rabbit antiserum directed at the 16 out-of-frame amino acids of the putative protein was produced. An ELISA assay was used to try to detect mutant BRCA2 protein, employing this antiserum.

In the first set of experiments, wells were coated with lysates of heterozygous fibroblasts on one hand, and with lysates of wt fibroblasts on the other. The synthetic peptide used for antibody generation was used as a control. As shown in Fig. 3a, no mutant protein was detected in this assay. Furthermore, when lysates of cells transfected with pIND-BRCA2 999del5 were tested in the same assay, they also failed to detect any mutant protein (Fig. 3b).

Detection of BRCA2 999del5 protein after proteasome inhibition

After all the different attempts to detect the mutant BRCA2 protein had failed, we had still not been able to demonstrate whether this was due to extremely rapid degradation of the protein or simply to a lack of sensitivity by our detection methods. To try to distinguish between these possibilities, we performed proteasome inhibitions of COS7 cells overexpressing BRCA2 999del5.

In the first set of proteasome inhibition experiments, the cells were inhibited for 0, 5, 10 and 20 hours, and cell lysis and protein purification were performed under non-denaturing conditions. Western blotting of p53 was used as a control for successful inhibition. No mutant BRCA2 protein was detectable in these samples (data not shown).
There is a possibility that the protein is missing from the lysate because the recombinant protein is folded in such a way that the V5 epitope and the poly-His tag are inaccessible to the α-V5 antibody and nickel-coated agarose beads, respectively. Therefore, in a new set of experiments, the cells were lysed and the proteins purified under denaturing conditions. No protein could be detected in the lysates or after purification with nickel when the cells were not treated with proteasome inhibitors (Fig. 4a). However, when the cells had been treated with lactacystin for 5 hours, a 39 kDa band (the expected size of recombinant BRCA2 999del5) emerged and showed a definite increase after 10 hours (Fig. 4b). These results show that even when overexpressed, the BRCA2 999del5 protein is rapidly degraded. It is therefore highly unlikely that this mutant protein has any functional effect in heterozygous individuals.
A large number of mutations in the \textit{BRCA2} gene have been identified to date that predispose to breast cancer, as well as to cancers in other organs. However, relatively few functional studies have been carried out on mutant \textit{BRCA2} proteins. In the case of truncating mutations, it is often assumed that these alleles give rise to unstable transcripts that are detected and eliminated by a nonsense-mediated mRNA decay pathway \cite{14, 15}. In the cases where residual nonsense transcripts escape this elimination process, they may not be available to the translation machinery because they are not trafficked to the cytoplasm or may not be properly assembled into a closed loop conformation, precluding efficient translation initiation \cite{6}. As a result, it has been generally accepted that most truncation mutants of the \textit{BRCA} genes lead to haploinsufficiency.

It should be noted, however, that this rule is not universal and that mutant \textit{BRCA} gene products may affect the oncogenic process. First, it has been firmly demonstrated that mutations in a 3.3 kb region in exon 11, termed the ovarian cancer cluster region, are preferentially associated with ovarian cancer rather than with breast cancer \cite{7, 17}. The mechanism underlying this difference in phenotype is not understood, but the observation that this region coincides with the RAD51-binding domain of \textit{BRCA2} \cite{18, 19} suggests that mutant proteins might be involved. Second, a few studies have examined the functional effect of overexpression of truncated \textit{BRCA} gene products. It was thus shown that several carboxyl-terminal truncated \textit{BRCA1} proteins conferred decreased susceptibility to apoptosis, impaired ability to suppress tumor growth \textit{in vivo} and blocked the function of ectopically expressed \textit{BRCA1} \cite{3}. Furthermore, a truncated \textit{BRCA1} mutant led to dominant negative effect of \textit{BRCA1}, as shown by an increase in both tumorigenicity \textit{in vivo} and chemosensitivity \cite{5}. Taken together, these observations suggest that it may be necessary to study each mutation separately at the cellular level.

In the present study, we examined mRNA and protein expression of a \textit{BRCA2} gene carrying an Icelandic founder mutation. Mutant mRNA represented about 20\% of the total \textit{BRCA2} transcript, demonstrating that the mutant message is present but is probably less stable than its normal counterpart. Notably, we were unable to find any traces of mutant protein product although several different techniques were employed.

Because negative results always leave open the possibility that the detection methods have failed, we went to considerable lengths in trying to overexpress mutant \textit{BRCA2} 999del5 protein in COS7 cells. \textit{BRCA2} 999del5 is predicted to contain the first 256 amino acids of wt \textit{BRCA2} followed by 16 out-of-frame amino acids, and to have a mass of around 35 kDa. This means that although the putative protein lacks the nuclear localization signal found at the carboxyl terminus of the protein \cite{2}, it would probably be able to diffuse passively into the nucleus because the cutoff mass for passive diffusion through the nuclear pore is 65 kDa \cite{20, 21}. This possibility sets \textit{BRCA2} 999del5 apart from larger known \textit{BRCA2} proteins.
mutants, which are kept in the cytoplasm due to the lack of nuclear localization signal.

We searched for the BRCA2 999del5 protein both in the cytoplasm and in the nucleus, but to no avail. Our results demonstrate that the BRCA2 999del5 encoded protein is extremely unstable and not detectable in cell lysates except under conditions that block degradation by proteasomes. This extreme instability of the protein makes it highly unlikely that it has any functional effect in heterozygous cells and supports strongly the fact that the increased cancer risk of BRCA2 999del5 carriers is solely due to haploinsufficiency.

Conclusions

In the present study, we asked whether the increased risk of cancer in BRCA2 999del5 carriers is simply due to haploinsufficiency or whether the gene produces a protein product that interferes with normal cellular processes. Although BRCA2 999del5 mRNA was detected in heterozygous cells, we found no evidence of mutant protein in cell lysates. Furthermore, overexpression studies showed that the BRCA2 999del5 protein is extremely unstable. BRCA2 999del5-associated cancer risk is therefore most probably due to haploinsufficiency.

Competing interests

None declared.

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