European Journal of Human Genetics (2003) 11, 951–954

www.nature.com/ejhg

ARTICLE

Alternatively spliced, truncated human *BRCA2* isoforms contain a novel coding exon

Marsha D Speevak*[,1,2], Sean S Young[1,3], Harriet Feilotter[4] and Peter Ainsworth[5]

[1]*Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, Ontario, Canada;* [2]*Department of Laboratory Medicine, The Credit Valley Hospital, Mississauga, Ontario, Canada;* [3]*Department of Pathology and Laboratory Medicine, Children's and Women's Health Centre of British Columbia, Vancouver, British Columbia, Canada;* [4]*Department of Pathology, Queens University, Kingston, Ontario, Canada;* [5]*LHSC Victoria Hospital Campus, London, Ontario, Canada*

The protein truncation test (PTT) employs *in vitro* transcription and translation of amplified cDNA and exonic gDNA to reveal truncating germ-line mutations. In a series of PTT analyses, abnormal splicing in the region encompassing exons 20–23 of *BRCA2* was discovered in leucocytes from high-risk breast cancer patients. Although sequencing of the genomic DNA in this region failed to reveal a detectable mutation in these patients, cDNA obtained from this region of *BRCA2* uncovered numerous alternative splice isoforms. PTT analysis and nested RT-PCR using RNA from leucocytes from healthy individuals, normal tissue and breast and ovarian cancer tumours demonstrated the presence of these alternatively spliced transcripts in all cases. The splice forms appeared to be more prominent in RNA from aged blood, suggesting that isoform expression was conditional. It is therefore important to distinguish naturally occurring alternative splicing from true splice defects due to mutations when interpreting PTT results.
European Journal of Human Genetics (2003) **11,** 951–954. doi:10.1038/sj.ejhg.5201063

Keywords: alternative splicing; BRCA2; PTT; tumour suppressor gene

Introduction

The breast cancer susceptibility gene *BRCA2* was first identified by positional cloning in 1995.[1] Subsequent family studies confirmed that *BRCA2* fit into the tumour suppressor gene classical model,[2,3] with a role in the maintenance of DNA integrity.[4,5] When initially isolated, the human *BRCA2* transcript was shown to consist of 27 exons, coding for 3418 amino acids.[1] Naturally occurring variants of *BRCA2* having altered regulatory activity have also been identified.[6,7] The presence of these variants has complicated the interpretation of diagnostic tests that rely on the detection of protein species. Here, we report new *BRCA2* isoforms first detected in leucocyte RNA from patients at high risk for a germ-line *BRCA* mutation.

***Correspondence: Dr MD Speevak, Department of Laboratory Medicine, Division of Genetics, The Credit Valley Hospital, 2200 Eglinton Ave. W, Mississauga, Ontario, Canada L5M 2N1. Tel: +1 905 813 4104; Fax: +1 905 813 4347; E-mail: mspeevak@cvh.on.ca**
Received 31 March 2003; revised 9 June 2003; accepted 18 June 2003

Materials and methods

Cells, cell lines and tissues

Patient peripheral blood samples were obtained from individuals with early-onset breast cancer and a family history of breast cancer. Normal control blood and tissue samples were obtained from anonymous, unrelated healthy individuals. Epithelial ovarian tumours were flash-frozen, anonymous, archived specimens. Cell lines were obtained from the American Type Culture Collection.

RNA and RT/PCR

RNA was isolated using RNeasy (Qiagen, Valencia, CA, USA) and Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA). Reverse transcription was performed with Superscript II Reverse Transcriptase (Invitrogen Life Technologies) using random hexamers, oligodT primers and 2 μg of DNAse I-treated, total RNA as template. The following primers were used: Exons 18–23 of *BRCA2* forward: p3274, 5′-$_{102593}$CTTGTTCTCTGTGTTTCTGAC-3′

or p3586: 5′-$_{102000}$GAAAGGGTGCTTCTTCAAC-3′. (*Note*: A T7 promoter and translation/initiation sequence was added 5′ to the unique primer sequence for the purpose of protein truncation test (PTT)). Exons 18–23 of *BRCA2* reverse: p3293, 5′-$_{119209}$TTTTTGTCGCTGCTAACTGTA-3′ or p3472, 5′$_{119366}$-TCCCGTGGCTGGTAAATCTGA-3′.

PCR conditions were as follows: 95°C 3 min, 5 × (95°C 60 s, 50°C 90 s, 72°C 90 s), 35 × (95°C 60 s, 47°C 90 s, 72°C 90 s), and one cycle of 72°C 10 min.

Seminested amplification of exon 20A-containing isoforms was performed on amplicons of exons 18–23 using reverse primer p3293, with an exon 20A-specific forward primer p850: 5′-$_{114673}$CACTGAAGTCTTGAACCCCC-3′.

PCR conditions were as follows: 95°C 3 min, 35 × (95°C 60 s, 55°C 90 s, 72°C 90 s) and one cycle of 72°C 10 min.

Note: Nucleotide position of primers is based on PAC HS214K23 (gb Z74739).[8]

PTT analysis

A 3.75 μl PCR product aliquot was combined with [^{35}S]methionine and the TNT/T7 coupled wheat germ system (Promega, Madison, WI, USA) according to the manufacturer's instructions. The protein products were separated on a 10% SDS-polyacrylamide minigel (PAGE) (BioRad, Hercules, CA, USA). The gels were fixed in 20% methanol/10% acetic acid, and dried at 80°C for 1 h followed by autoradiography.

Subcloning and sequencing of PCR products

DNA products from the amplification of exons 18–23 were cloned into PCR2.1 (Invitrogen Life Technologies) and end sequenced using universal primers. Alternatively, an M13-tailed reverse primer was used in the amplification of noncloned cDNA from the same region (exons 18–21) and sequenced using an M13 universal primer by automated sequence analysis (Applied Biosystems, Foster City, CA, USA; Visible Genetics, Toronto, ON, USA).

Results

Identification of new *BRCA2* isoforms

A research project employing PTT to scan for *BRCA2* mutations in sporadic ovarian tumours uncovered a consistent difference between ovarian tumour and leucocyte PTT patterns produced from *BRCA2* exons 18–23. Ovarian tumour PTT gels had a prominent band in the expected size range for the cDNA amplicon, at approximately 46.9 kDa (Fragment A, Figure 1). However, leucocyte samples from patients at high risk for familial breast cancer displayed a second prominent PTT band of approximately 28.3 kDa (Fragment B, Figure 1). To test whether Fragment B was a product of leucocyte specific alternative splicing, the PCR products obtained from amplification of exons 18–23 from the leucocytes of a breast cancer patient were subcloned and sequenced.

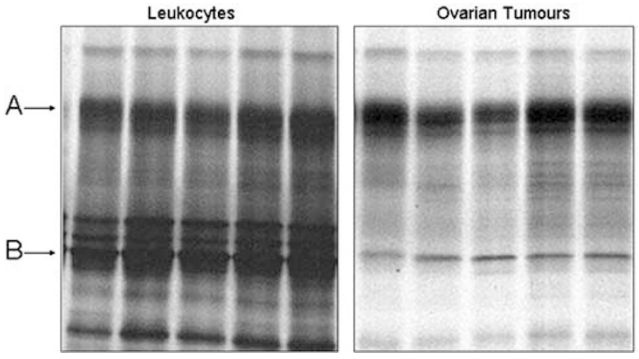

Figure 1 PTT analysis of exons 18–23 of *BRCA2*. SDS-PAGE gels showing PTT pattern obtained from leucocytes and ovarian tumour samples.

Several *BRCA2* isoforms were identified, each containing a 64 bp in-frame insertion between exons 20 and 21. These were collectively called BRCA2_v20 isoforms.

A BLAST search revealed that the insertion was a cryptic exon derived from *BRCA2* IVS 20 (nucleotides 114 658–114 721, PAC HS214K23[8]). This exon, which we called exon 20A, encoded 21 amino acids ending with a nonsense codon at the junction with exon 21. The sequence of the insertion was $_{8632}$G_*TTACTTCCTCCACTGAAGTCTTGAACC CCCCAA AGTCATCCATGAGGGTTGGAATCAACTTCTG*_A$_{8633}$, translating to amino acid sequence: VTSSTEVLNPPKSSMRV GINFX.

In addition, two isoforms eliminated exon 22 by utilizing a cryptic splice site in exon 23 at nucleotide position 9232. All isoforms were predicted to produce the same truncated protein lacking the amino acids encoded by the last seven exons of the gene.

Figure 2 shows the BRCA2_v20 variants. RNA species 'A' conformed to the published cDNA sequence for exons 18–23 of *BRCA2*. Isoforms v20B–v20E all contained exon 20A, but showed alternative splicing of exons 22 and 23. The PTT fragment obtained from primer set p3274/p3293 was predicted to produce a protein product of 345 amino acids (species 'A'). The theoretical size of the product produced by the alternative isoforms was 213 amino acids, resulting in a size ratio of 0.62:1 for the alternative isoforms relative to species 'A'. This was in agreement with the observed 0.60:1 size ratio of PTT Fragment B relative to PTT Fragment A (Figure 1).

Expression of BRCA2 isoforms

In order to determine if the isoforms were expressed in leucocytes from normal individuals, seminested PCR using an internal forward primer (p850) designed to amplify exon 20A-containing isoforms only was performed on exon 18–23 cDNA amplicons from 19 healthy individuals. First round PCR produced a blurred band in the 1100 bp range, as expected, since the various *BRCA2* amplicons

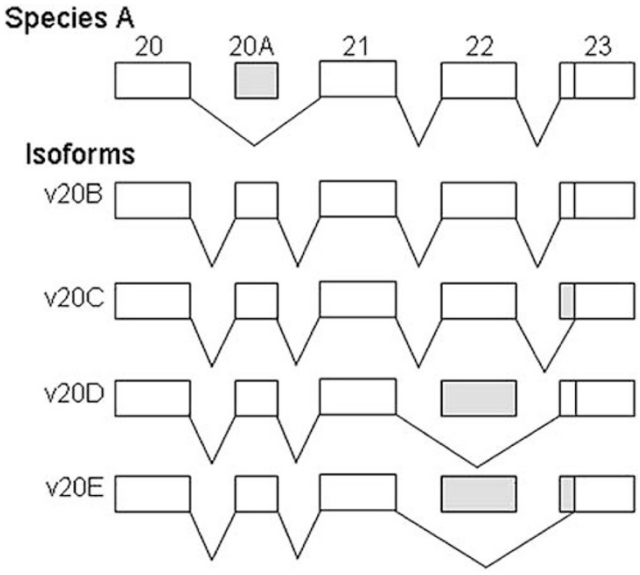

Figure 2 Diagram showing isoform structure. The numbers above the boxes indicate the individual exons and the shaded boxes represent regions eliminated via alternative splicing.

range in size between 889 and 1139 bp (not shown). Seminested PCR produced several electrophoretic bands in all lanes, corresponding in size to each of the BRCA2_v20 isoforms (Figure 3). The most prevalent band corresponded in size to the v20D isoform. Similar analyses using total RNA from normal breast and ovarian tissues, ovarian tumours and tumour cell lines (16 examined in total) demonstrated a single sharp band, corresponding in size to species A in the first round (data not shown). This agreed with the PTT results obtained from ovarian tissues that showed a single prominent band (Figure 1). Second round PCR using the exon 20A-specific primer set, which excluded species A, revealed a single major band consistent in size with isoform v20B (514 bp) in all lanes (Figure 3).

Detection of overexpressed exon 20A in breast cancer patients

In two independent, collaborating clinical laboratories, mutational analysis of *BRCA2* was performed routinely by PTT. The PTT pattern for the region containing exons 18–23 showed a secondary band consistent with the band seen in the leucocyte samples used in the ovarian tumour experiments. However, several unrelated patients appeared to have a stronger secondary band in the same position, interpreted to be a possible truncation. Electrophoresis of the PCR products revealed a second prominent PCR product of lower molecular weight, suggesting a splicing defect. Sequencing of the cDNA from these patients revealed a 64 bp insertion between exons 20 and 21 identical to that cloned in the research laboratory. In these

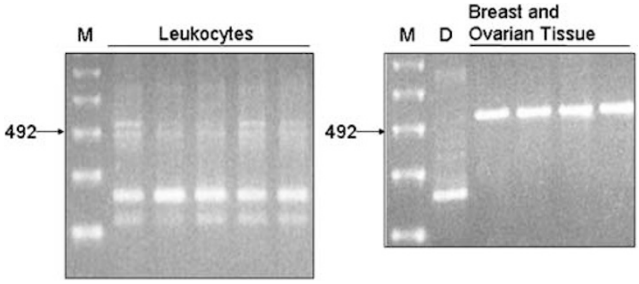

Figure 3 Seminested RT-PCR amplification of exons 18–23. Left: Examples of second round PCR amplification of leucocytes from normal healthy controls using primers specific for the v20 isoforms: v20B: 514 bp; v20C, 463 bp; v20D, 315 bp (predominant); v20E, 264 bp. Right: Examples of second round PCR amplification of tissue from breast and ovarian samples expressing v20B only. M – 123 bp DNA ladder; D – cloned v20D (315 bp).

cases, genomic sequencing failed to reveal any mutations in the region.

A review of the clinical leucocyte specimens revealed that PTT Fragment B was strongest in blood samples that had been stored at room temperature over a period of at least 24 h. Fresh samples were obtained from the patients for repeat analysis by PTT. PTT experiments performed using fresh RNA resulted in a diminished Fragment B, confirming the suspicion that the previous samples had been affected by conditional alternative splicing.

Discussion

While nonsense-mediated mRNA decay may cause false-negative results in PTT experiments,[9] false-positive results due to illegitimate splicing have not been previously described as a possible pitfall of PTT. In our series of PTT analyses, the *BRCA2* splice variants were observed in both patient and control tissues. Also, no genomic mutations that might explain the alternative splicing were found in any of the samples exhibiting increased isoform expression. Since overexpression of the v20 variants was most evident in aged blood samples, it likely represented a response to environmental stress. At least three distinct illegitimate splice forms arising from aged blood samples have also been found in the 3′ end of *BRCA1* (data not shown). Therefore, the possibility of false-positive PTT results for the 3′ region of *BRCA2* as well as *BRCA1* must be considered when interpreting test results.

The prominence of frameshift and premature stop codons in *BRCA2* mutation families suggests that *BRCA2* truncating mutations are important in breast cancer susceptibility. Also, the involvement of the 3′ terminus of *BRCA2* in DNA repair pathways and protein localization has been proven experimentally.[10–12] Differences in the

range of expression of individual v20 splice variants in breast- and ovarian-derived tissues as compared to leucocytes suggested physiological tissue specificity and not just illegitimate splicing alone. It was therefore important to evaluate the expression patterns of the newly discovered alternative *BRCA2* isoforms to determine if they were of aetiologic significance to high-risk breast cancer patients. Heterogeneous expression of *BRCA2*, as described here, appears to be similar to the temperature-dependent expression and tissue-based variability seen in the *NF1* message.[13–15] Tumour suppressor genes *TP53* and *APC* also appear to undergo similar, unexplained, truncating alternative splicing.[16,17] Acquired defects in pre-mRNA processing have been proposed as a factor in causing and predisposing individuals to disease.[18] Since tumour suppressor genes are acutely susceptible to inactivation through truncation events, it is possible that epigenetic or environmental factors leading to increased expression of truncating isoforms in sensitive tissues impact on disease progression.

When characterizing the expression of tumour suppressor genes *in vitro* or when evaluating patient samples for expression abnormalities, it is important to rule out conditional alternative splicing as a possible mechanism of abnormal expression. An increased understanding of the conditions leading to alternative splicing in clinical samples is also extremely important so that compromised interpretation of RNA-based analyses is avoided.

Acknowledgements

We thank RG Korneluk for his mentorship and support of SS Young and MD Speevak. We also thank M Fung Kee Fung and M Senterman for generously providing the ovarian tumour tissues.

References

1 Wooster R, Bignell G, Lancaster J *et al*: Identification of the breast cancer susceptibility gene BRCA2. *Nature* 1995; **378**: 789–792.
2 Collins N, McManus R, Wooster R *et al*: Consistent loss of the wild type allele in breast cancers from a family linked to the BRCA2 gene on chromosome 13q12–13. *Oncogene* 1995; **10**: 1673–1675.
3 Gudmundsson J, Johannesdottir G, Bergthorsson JT *et al*: Different tumor types from BRCA2 carriers show wild-type chromosome deletions on 13q12–q13. *Cancer Res* 1995; **55**: 4830–4832.
4 Wong AKC, Pero R, Ormonde PA, Tavtigian SV, Bartel PL: RAD51 interacts with the evolutionarily conserved BRC motifs in the human breast cancer susceptibility gene brca2. *J Biol Chem* 1997; **272**: 31941–31944.
5 Marmorstein LY, Ouchi T, Aaronson SA: The BRCA2 gene product functionally interacts with p53 and RAD51. *Proc Natl Acad Sci USA* 1998; **95**: 13869–13874.
6 Zou JP, Yoshinbu H, Siddique H, Rao VN, Reddy ESP: Structure and expression of variant BRCA2a lacking the transactivation domain. *Oncol Rep* 1999; **6**: 437–440.
7 Bieche I, Lidereau R: Increased level of exon 12 alternatively spliced BRCA2 transcripts in tumor breast tissue compared with normal tissue. *Cancer Res* 1999; **59**: 2546–2550.
8 Hunt S: Human DNA sequence from clone RP1-214K23 on chromosome 13, containing BRCA2 gene. NCBI Entrez database 2001; gi|1929047|emb|Z74739.1|HS214K23 [1929047].
9 Garvin AM: A complete protein truncation test for BRCA1 and BRCA2. *Eur J Hum Genet* 1998; **6**: 226–234.
10 Connor F, Bertwistle D, Mee PJ *et al*: Tumorigenesis and a DNA repair defect in mice with a truncating *Brca2* mutation. *Nat Genet* 1997; **17**: 423–430.
11 Spain BH, Larson CJ, Shihabuddin LS, Gage FH, Verma IM: BRCA2 is cytoplasmic: implications for cancer-linked mutations. *Proc Natl Acad Sci USA* 1999; **96**: 13920–13925.
12 Yano K, Morotomi K, Saito H, Kato M, Matsuo F, Mini Y: Nuclear localization signals of the BRCA2 protein. *Biochem Biophys Res Commun* 2000; **270**: 171–175.
13 Murtif-Park V, Kenwright KA, Surtevant DB, Karman Pivnick E: Alternative splicing of exons 29 and 30 in the neurofibromatosis type 1 gene. *Hum Genet* 1998; **103**: 382–385.
14 Wimmer K, Eckart M, Rehder H, Fonatsch C: Illegitimate splicing of the NF1 gene in healthy individuals mimics mutation-induced splicing alterations in NF1 patients. *Hum Genet* 2000; **106**: 311–313.
15 Thomson SA, Wallace MR: RT-PCR splicing analysis of the NF1 open reading frame. *Hum Genet* 2002; **110**: 495–502.
16 Flaman JM, Waridel F, Estreicher A *et al*: The human tumor suppressor gene p53 is alternatively spliced in normal cells. *Oncogene* 1996; **12**: 813–818.
17 Sulekova Z, Reina-Sanchez J, Ballhausen WG: Multiple APC messenger RNA isoforms encoding exon 15 short open reading frames are expressed in the context of a novel exon 10A-derived sequence. *Int J Cancer* 1995; **63**: 435–441.
18 Stoilov P, Meshorer E, Gencheva M, Glick D, Soreq H, Stamm S: Defects in pre-mRNA processing as causes of and predisposition to diseases. *DNA Cell Biol* 2002; **21**: 803–818.