Low incidence of *BRCA2* mutations in breast carcinoma and other cancers

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Inherited mutant alleles of familial tumour suppressor genes predispose individuals to particular types of cancer. In addition to an involvement in inherited susceptibility to cancer, these tumour suppressor genes are targets for somatic mutations in sporadic cancers of the same type found in the familial forms[1]. An exception is *BRCA1*, which contributes to a significant fraction of familial breast and ovarian cancer, but undergoes mutation at very low rates in sporadic breast and ovarian cancers[2–4]. This finding suggests that other genes may be the principal targets for somatic mutation in breast carcinoma. A second, recently identified familial breast cancer gene, *BRCA2* (refs 5–8), accounts for a proportion of breast cancer roughly equal to *BRCA1*. Like *BRCA1*, *BRCA2* behaves as a dominantly inherited tumour suppressor gene. Individuals who inherit one mutant allele are at increased risk for breast cancer, and the tumours they develop lose the wild-type allele by heterozygous deletion[9]. The *BRCA2* coding sequence is huge, composed of 26 exons that span 10,443 bp[8]. Here we investigate the rate of *BRCA2* mutation in sporadic breast cancers and in a set of cell lines that represent twelve other tumour types. Surprisingly, mutations in *BRCA2* are infrequent in cancers including breast carcinoma. However, a probable germline mutation in a pancreatic tumour cell line suggests a role for *BRCA2* in susceptibility to pancreatic cancer.

Tumour suppressor genes can be inactivated by homozygous deletion; indeed, detection of a homozygous deletion in a pancreatic xenograft was instrumental in the effort to isolate *BRCA2* by positional cloning[10]. The chance observation of a *BRCA2* homozygous deletion suggested that other events might occur in breast tumours or in additional cancer types. We screened a set of 150 cell lines derived from different cancers for homozygous deletions in the vicini-

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ty of *BRCA2*. Sixteen sequence-tagged sites (STSs) spanning about 650 kb of genomic DNA were used in PCR experiments to test for amplification from genomic DNA (Fig. 1). Reproducible absence of amplification by particular primer pairs suggested homozygous deletion; the use of cell lines facilitated the analysis since primary specimens often contain some normal tissue that readily supports amplification[11]. We identified homozygous deletions in three cell lines — two colon (LS174 and SW620) and one breast (BT20) — by failure of a single STS (B52FT7) to amplify, use of several overlapping STSs confirmed this result. The deletion boundaries appeared identical in the three lines and were located roughly 6 kb apart. The deleted region lies nearly 350 kb 5' of the *BRCA2* transcriptional start site and is separated from *BRCA2* by at least one other gene (S. Tavtigian, unpublished data), and is, thus, unlikely to interfere with *BRCA2* function. In addition, the deletion appears to be a common polymorphism based on analysis of normal DNAs using a deletion-specific primer pair as a probe (see Methods). The homozygous deletion detected in the cell lines was the result of either loss of heterozygosity (LOH) on one homolog, which uncovered the deletion (BT20 and LS174), or homozygosity of the polymorphic deleted chromosome (SW620).

Having found no evidence for homozygous deletion of *BRCA2* in our experiments, we investigated whether smaller lesions such as point mutations contributed to *BRCA2* inactivation. Since compound mutant heterozygotes and mutant homozygotes are rare, tumour suppressor gene inactivation nearly always involves LOH. The remaining allele, if inactive, typically contains disruptive mutations. Thus, if the goal is to identify mutations in tumour suppressor genes, it is sensible to preselect tumours or cell lines that exhibit LOH at the locus of interest[2].

We examined a group of 104 primary breast tumour samples and a set of 269 cell lines for LOH in the *BRCA2* region. For the primary tumours, we quantitatively compared amplifications of three short tandem repeat markers (STR4247, STR257, STR561A) using fluorescence. Based on their combined heterozygosity indices, the chance that these markers will all be homozygous in a particular individual (assuming
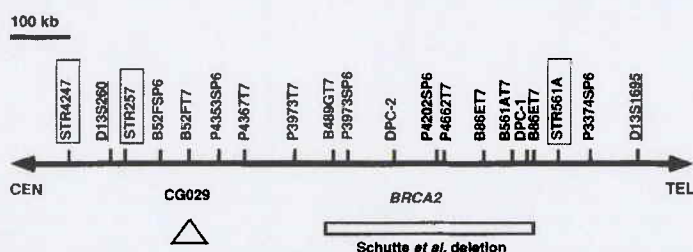

Fig. 1 Screen for homozygous deletions in tumour cell lines. The positions of STS markers used in the analysis are shown. *D13S260* and *D13S165*, STR markers that help orient the physical and the genetic maps, are underlined. The three STRs used to determine LOH are boxed. Sequences of the STS primers are available upon request. In an earlier search for genes within this region, several cDNA fragments were joined together into a candidate gene (*CG029*) that includes sequences within the deletion (S. Tavtigian, unpublished data). No splice junctions were identified within *CG029*, but a probe recognized predominantly a 2.4-kb mRNA species from testis and heart on a poly(A)+ northern blot. The locations and transcription orientations of *BRCA2* and *CG029* are shown by arrows. The deletion polymorphism location is shown by a triangle. Also shown is the extent of the homozygous deletion identified in a pancreatic xenograft[10]. CEN, centromere; TEL, telomere.

Fig. 2 LOH analysis of primary breast tumours. Alleles of STR markers are indicated below the chromatogram. a, Example of a tumour heterozygous at *BRCA2*. b, Example of a tumour with LOH at *BRCA2*. Fluorescence units are on the ordinate; size in basepairs is on the abcissa. T, tumour; N, normal.
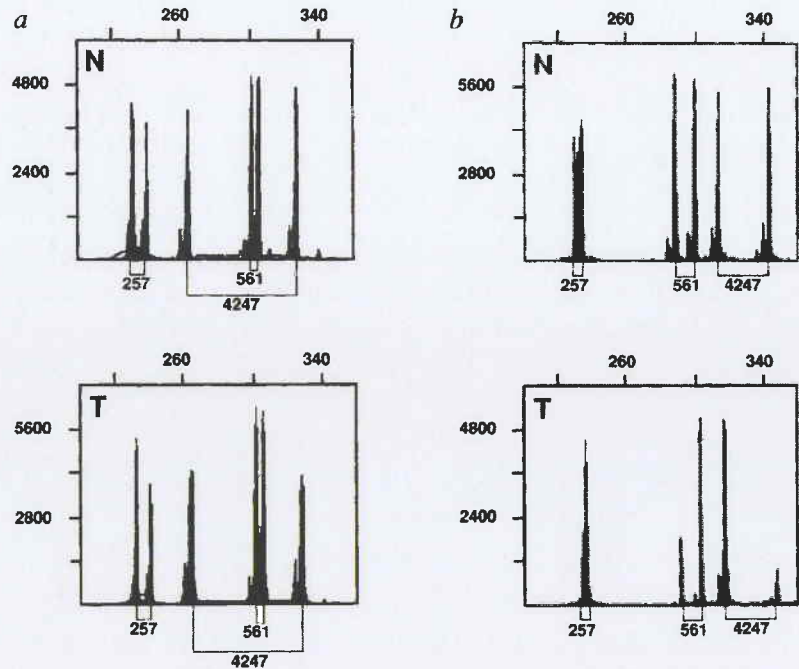
linkage equilibrium) is only one in 250. Even if there is LOH, the presence of normal cells in the primary tumour sample makes it impossible to entirely eliminate signal from the lost allele. Rather, the relative intensities of the two alleles are altered as can clearly be seen by comparing the allelic peak heights from normal tissue with peak heights from the tumour (Fig. 2). Using this analysis, we classified 30 tumours (29%) as having LOH at the *BRCA2* locus (Table 1); this figure is similar to previous estimates[9,12].

We assessed LOH in the cell lines in a different fashion. Since homozygosity of all three STRs together in a single sample was improbable, and since normal cells were not present, we interpreted apparent homozygosity at all STRs as LOH in the *BRCA2* region. Using this criterion, 85/269 of the cell lines exhibited LOH (Table 1). The frequencies in lines of different tumour types varied: 4/6 ovarian cell lines and 31/62 lung cancer lines displayed LOH compared with 17/81 melanoma lines and 2/11 breast cancer lines.

We screened the group of 30 primary breast cancers, preselected for LOH in the *BRCA2* region (Table 1), by DNA sequence analysis for sequence variants. We examined greater than 95% of the coding sequence and splice junctions of *BRCA2* in each sample. One specimen contained a frameshift mutation, one a missense and a frameshift mutation, one a nonsense mutation, and one a missense mutation (Table 2a). The nonsense mutation would delete 156 codons at the C terminus, suggesting that the C-terminal end of *BRCA2* is important for tumour suppressor activity. All sequence variants were also present in the corresponding normal DNA from these cancer patients indicating that these were germline lesions. To exclude the unlikely possibility that preselection for LOH introduced a systematic bias against detecting mutations (for example, dominant mutations or compound heterozygotes), 12 samples heterozygous at *BRCA2* were also screened. Three of these revealed missense changes that were also found in the corresponding normal samples. Thus, in a set of 42 breast carcinoma samples, 30 of which displayed LOH at the *BRCA2* locus, no somatic mutations were identified. The frameshift and nonsense changes are likely to be predisposing mutations that influenced development of breast cancer in these patients. The missense variants are rare; they were each observed only once during analysis of 115 chromosomes. From these data, however, it is not possible to distinguish between rare neutral polymorphisms and predisposing mutations.

Table 1 LOH analysis of cell lines and primary breast tumours			
Type	Number LOH /screened	Percentage LOH	Number sequenced
Astrocytoma	6 / 19	32%	6
Bladder	6 / 17	35%	4
Breast	2 / 11	18%	2
Colon	2 / 8	25%	2
Glioma	11 / 36	31%	5
Lung	31 / 62	50%	20
Lymphoma	0 / 4	0	0
Melanoma	17 / 81	21%	9
Neuroblastoma	1 / 10	10%	1
Ovarian	4 / 6	67%	4
Pancreatic	1 / 3	33%	1
Prostate	0 / 2	0	0
Renal	4 / 10	40%	4
Total	85 / 269	33% (avg. = 28%)	58
Primary breast	30 / 104	29%	42

Percentage LOH was calculated two ways: as total and as a mean of percentages (avg.).

Table 2a Mutations identified in *BRCA2*

Sample	Type	LOH	Change	Effect	Germline
4H5	Renal	yes	G451C	Ala→Pro	n.d.
4G1	Ovarian	yes	A1093C	Asn→His	n.d.
2F8	Lung	yes	G1291C	Val→Leu	n.d.
BT110	Primary breast	yes	1493delA	frameshift	yes
4F8	Ovarian	yes	C2117T	Thr→Ile	n.d.
BT163	Primary breast	no	A2411C	Asp→Ala	yes
1D6	Bladder	no	G4813A	Gly→Arg	n.d.
BT333	Primary breast	no	T5868G	Asn→Lys	yes
BT111	Primary breast	yes	6174delT	frameshift	yes
4G3	Pancreatic	yes	as above	as above	n.d.
1B7	Astrocytoma	yes	C6328T	Arg→Cys	n.d.
BT118	Primary breast	no	G7049T	Gly→Val	yes
BT115	Primary breast	yes	G7491C	Gln→His	yes
3D5	Melanoma	yes	A9537G	Ile→Met	n.d.
BT85	Primary breast	yes	A10204T	Lys→stop	yes
1E4	Breast	yes	C10298G	Thr→Arg	n.d.
BT110	Primary breast	yes	A10462G	Ile→Val	yes

Listed are the mutation positions based on the Genbank entry of *BRCA2* (Accession number HSU43746)[8]. 'As above' means that the tumour has the same change as the sample listed before it. n.d., not determined.

Table 2b Common polymorphisms and silent substitutions detected in *BRCA2* by DNA sequencing

Position	Change	Effect	Frequency
5'UTR(203)	G/A	–	0.32 (0.26)
PM(1342)	A/C	Asn→His	0.32 (0.37)
PM(2457)	T/C	silent	0.04 (0.05)
PM(3199)	A/G	Asn→Asp	0.04 (0.08)
PM(3624)	A/G	silent	0.35
PM(3668)	A/G	Asn→Ser	0 (0.15)
PM(4035)	T/C	silent	0.24 (0.10)
PM(5972)	C/T	Thr→Met	0.03
PM(7470)	A/G	silent	0.26 (0.15)
1593	A→G	silent	<0.01
4296	G→A	silent	<0.01
5691	A→G	silent	<0.01
6051	A→G	silent	<0.01
6828	T→C	silent	<0.01
6921	T→C	silent	<0.01

Since some rare silent variants may affect gene function (for example, splicing)[21], these are not preceded by "PM". The frequencies of polymorphisms shown involve the second of the nucleotide pair. Frequencies reported in a previous study are shown in parentheses[8]. Numbering is as in Table 2a.

The technical ease with which sequence changes can be detected in cell lines, and the opportunity to examine *BRCA2* in cell lines derived from tumours other than breast, led us to screen a group of 58 cell lines preselected for LOH at *BRCA2* (Table 1). We screened greater than 95% of the *BRCA2* coding sequence of each sample, but only one clearly disruptive mutation, a frameshift, was identified by DNA sequence analysis (Table 2a). This mutation (6174delT) was present in a pancreatic cancer line and is identical to one found in the germline of primary tumour, BT111, and to previously detected germline frameshift lesions[8,13]. These findings suggest that this particular frameshift may be a relatively common germline *BRCA2* mutation. In addition, eight missense sequence variants were detected (Table 2a); however, it was not possible to determine whether these sequence variants were somatic and whether they compromise *BRCA2* protein function.

Detection of a probable germline *BRCA2* mutation in a pancreatic tumour cell line suggests that *BRCA2* mutations may predispose to pancreatic cancer. This mutation also adds weight to the involvement of *BRCA2* in sporadic pancreatic cancer, implied previously by the homozygous deletion observed in a pancreatic xenograft[10]. Since we examined only three pancreatic cell lines, further investigation of *BRCA2* mutations in pancreatic cancers is warranted.

The lack of evidence for somatic inactivation places *BRCA2* in a select company of familial tumour suppressor genes that are infrequently mutated in sporadic tumours. Among tumour suppressor genes identified through kindred studies so far, only *BRCA1*, *WT1* and possibly *hMLH1* and *hMSH2* exhibit such features. It is conceivable that modes of inactivation other than deletion and mutation, such as methylation-silencing, may occur[14,15]. However, in no case has methylation been identified as the sole mechanism for inactivation of a specific tumour suppressor gene. It is also unlikely that a large number of mutations were missed since we screened nearly the entire coding sequence in every sample and detected polymorphisms at frequencies consistent with previous work (Table 2b). In addition, most germline *BRCA2* mutations described so far are of the frameshift variety[7,8]. These are especially easy to spot. Thus, it is probable that *BRCA2* is not inactivated at appreciable frequencies in sporadic breast tumours, despite its prominent role in hereditary breast cancer.

The observation, however, that roughly a third of breast tumours and tumour cell lines have lost one copy of *BRCA2* may suggest a role for another tumour suppressor located nearby on chromosome 13. The retinoblastoma locus, *RB1*, lies on the short arm of chromosome 13 and may be responsible for some LOH, particularly in lung cancer lines[16,17]. In addition, random chromosome deletion may contribute to LOH in the region as described for other chromosomal locations[18,19]. As was the case with *BRCA1*, attempts to use LOH in sporadic breast tumours to localize *BRCA2* would have given false information[20].

The hope that study of familial cancer genes will yield insight into tumour progression has been amply justified. In the case of breast carcinoma, the spectrum of genes influencing predisposition through germline mutation may be different from the genes inactivated by somatic mutation during progression. Nevertheless, studies of *BRCA1* and *BRCA2* provide the opportunity to gain fundamental insights into growth control pathways that operate within breast epithelial cells.

Methods

DNA preparation. Primary breast tumour and normal sample pairs were anonymously obtained from the Memorial Sloan-Kettering Cancer Center. Patient histories are not available, but the sample is random, unselected for age of onset or family history. Cancer cell lines were purchased from the ATCC. DNA from cell lines was prepared as described[19]. DNA from tissue was isolated using the Easy-DNA kit (Invitrogen).

PCR amplification. A total of 33 amplicons were generated by PCR that encompass the 26 coding exons of *BRCA2*. Nested primer sets were used for all amplifications. In general, the PCR conditions were: an initial single denaturation step at 95 °C for 1 min followed by cycles of denaturing at 95 °C (6 s), annealing at 55 °C (15 s) and extension at 72 °C (1 min). For the primary amplification step, 1 to 10 ng of genomic DNA

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were subjected to a 20 cycle reaction after which the primary PCR products were diluted ten-fold and reamplified using nested primers for another 33 cycles.

DNA sequencing and mutation screening. DNA sequencing was carried out either on the ABI 377 (Applied Biosystems Division, Perkin-Elmer) or manually. For the radioactive mutation screen, the amplified products were purified on agarose gels followed by Qiaquick (Qiagen). DNA sequence was generated using the Cyclist sequencing kit (Stratagene) and resolved on 6% polyacrylamide gels. In parallel, non-radioactive sequencing using fluorescent labelling dyes was performed using the TaqFS sequencing kit followed by electrophoresis on ABI 377 sequencers. Samples were gridded into 96-well trays to facilitate PCR and sequencing. Dropouts of particular PCR and sequencing reactions were repeated until >95% coverage was obtained for every sample. Sequence data were analysed with the Sequencher software (Genes Codes). All detected mutations were confirmed by sequencing a newly amplified PCR product to exclude the possibility of PCR artefacts. Primers for PCR and sequencing were in roughly half the cases identical to those used previously and are available upon request[8].

Loss of heterozygosity analysis. For genotyping, ~10 ng of genomic DNA was amplified by PCR with the following three sets of fluorescently tagged STRs (5'→3'):

(1)	mM4247.4A.2F1	ACCATCAAACACATCATCC
	mM4247.4A.2R2	AGAAAGTAACTTGGAGGGAG
(2)	STR257-FC	CTCCTGAAACTGTTCCCTTGG
	STR257-RD	TAATGGTGCTGGGATATTTGG
(3)	mMB561A-3.1FA2	GAATGTCGAAGAGCTTGTC
	mMB561A-3.1RB	AAACATACGCTTAGCCAGAC

The PCR products were resolved using the ABI377 and

quantified with Genescan software. For tumours, clear peak height differences between alleles amplified from normal and tumour samples were scored as having LOH. For cell lines, if one STR was heterozygous, the sample was scored as non-LOH. In only one case was a cell line or tumour miscalled based on later analysis of single base polymorphisms. The heterozygosity indices for the markers are: STR4247 = 0.89; STR257 = 0.72; STR561A = 0.88 (S. Neuhausen, personal communication; B. Swedlund, unpublished data).

Analysis of deletion polymorphism. A deletion-specific primer pair and a wild type-specific pair was used to assess the frequency of the polymorphism in a set of 87 control germline DNA samples. Only one sample appeared homozygous, whereas seven were heterozygous; thus, the overall deletion frequency is 5% of chromosomes. The primer pairs used were R130-A19 which generates a 3-kb product from wild-type genomic DNA, and R115-A19 which amplifies across the deleted region to yield a 2-kb fragment. The primer sequences were (5'→3'):

4353.R115	GGC ACC TAT ATC CAC AGA CA
4353.R130	AAG ACT CCT GGA GTC TAG AAC
4353.A19	CCA AAG ATT TAG TGT AAG CAG AAC

1. Knudson, A. G. All in the (cancer) family. *Nature Genet.* **5**, 103–104 (1993).
2. Futreal, P. A. *et al.* BRCA1 mutations in primary breast and ovarian carcinomas. *Science* **266**, 120–122 (1994).
3. Hosking, L. *et al.* A somatic *BRCA1* mutation in an ovarian tumor. *Nature Genet.* **9**, 343–344 (1995).
4. Merajver, S. D. *et al.* Somatic mutations in the *BRCA1* gene in sporadic ovarian tumor. *Nature Genet.* **9**, 439–443 (1995).
5. Wooster, R. *et al.* Localization of a breast cancer susceptibility gene, BRCA2, to chromosome 13q12–13. *Science* **265**, 2088–2090 (1994).
6. Thorlacius, S. *et al.* Linkage to BRCA2 region in hereditary male breast cancer. *Lancet* **346**, 544–545 (1995).
7. Wooster, R. *et al.* Identification of the breast cancer susceptibility gene *BRCA2. Nature* **378**, 789–792 (1995).
8. Tavtigian, S. V. *et al.* The complete *BRCA2* gene and mutations in chromosome 13q-linked kindreds. *Nature Genet.* **12**, 1–6 (1996).
9. Collins, N. *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* **10**, 1673–1675 (1995).
10. Schutte, M. *et al.* Identification by representational difference analysis of a homozygous deletion in pancreatic carcinoma that lies within the BRCA2 region. *Proc. Natl. Acad. Sci. USA* **92**, 5950–5954, (1995).
11. Kamb, A. et al. Response to Rates of p16 (MTS1) Mutations in Primary Tumors with 9p Loss. Cairns et al. 1995 *Science* **265**, 416–417 (1994).
12. Cleton-Jansen, A. M. *et al.* Loss of heterozygosity in sporadic breast tumours at the BRCA2 locus on chromosome 13q12–q13. *Br. J. Cancer*

72, 1241–1244 (1995).
13. Neuhausen, S. *et al.* Recurrent *BRCA2* 6174delT mutations in Ashkenazi Jewish women affected by breast cancer. *Nature Genet.* **13**, 126–128 (1996).
14. Gonzalez-Zulueta, M. *et al.* Methylation of the 5' CpG island of the *p16/CDKN2* tumor suppressor gene in normal and a transformed human tissues correlates with gene silencing. *Cancer Res.* **55**, 4531–4535 (1995).
15. Merlo, A. *et al.* 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor *p16/CDKN2/MTS1* in human cancers. *Nature Med.* **1**, 686–691 (1995).
16. Yokota, J. *et al.* Altered expression of the retinoblastoma (RB) gene in small-cell carcinoma of the lung. *Oncogene* **3**, 471–475 (1988).
17. Hensel, C. H. *et al.* Altered structure and expression of the human retinoblastoma susceptibility gene in small cell lung cancer. *Cancer Res.* **50**, 3067–3072 (1990).
18. Vogelstein, B. *et al.* Allelotype of colorectal carcinomas. *Science* **244**, 207–211 (1989).
19. Liu, Q. *et al.* CDKN2 (MTS1) tumor suppressor gene mutations in human tumor cell lines. *Oncogene* **10**, 1061–1067 (1995).
20. Emi, M. *et al.* A novel metalloprotease/disintegrin-like gene at 17q21.3 is somatically rearranged in two primary breast cancers, *Nature Genet.* **5**, 151–157 (1993).
21. Richard, I. & Beckmann, J. S. How neutral are synonymous codon mutations? *Nature Genet.* **10**, 259 (1995).