

ORIGINAL ARTICLE

Molecular evidence for putative tumour suppressor genes on chromosome 13q specific to BRCA1 related ovarian and fallopian tube cancer

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Background/Aims: Loss of heterozygosity (LOH) on chromosome 13q has been reported to occur frequently in human ovarian cancer, and indications have been found that chromosome 13 may also play a specific role in the inherited form of ovarian cancer. The aim of this study was to define regions on chromosome 13 that may harbour additional tumour suppressor genes involved in the tumorigenesis of BRCA1 related ovarian and fallopian tube cancer.

Materials/methods: DNA extracted from paraffin wax blocks of 36 BRCA1 associated ovarian and fallopian tube carcinomas was analysed by LOH polymerase chain reaction using seven highly polymorphic microsatellite markers spanning chromosome 13q.

Results: High LOH frequencies were found on loci 13q11, 13q14, 13q21, 13q22–31, 13q32, and 13q32–4, suggesting the presence of putative tumour suppressor genes on the long arm of chromosome 13 that may play a role in the pathogenesis of BRCA1 related ovarian and fallopian tube cancer. LOH patterns appeared to be independent of the type of BRCA1 mutation, stage, and grade. Although in some cases there were indications for loss of larger parts of chromosome 13, in most cases losses were fairly randomly distributed over chromosome 13 with retained parts in between lost parts. Microsatellite instability was found in six cases.

Conclusion: Several loci on chromosome 13q show high frequencies of LOH in BRCA1 related ovarian and fallopian tube cancer, and may therefore harbour putative tumour suppressor genes involved in the carcinogenesis of this particular type of hereditary cancer.

Ovarian cancer is the fifth most common cancer in women and the most common cause of death from gynaecological malignancy in the Western world.^{1–3} It has been estimated that 5–10% of ovarian epithelial cancer is inherited in a dominant autosomal fashion, mostly associated with germline mutations in the BRCA1 or BRCA2 genes.^{6–11} The penetrance of ovarian cancer among BRCA1 mutation carriers was originally estimated at 45% at the age of 70, and that of breast cancer at about 85%.¹² In fact, the cancer risk among BRCA mutation carriers may vary. For example, in a study in Jewish subjects the risks for carriers of the most common founder mutations are estimated at 16% for ovarian carcinoma and 56% for breast carcinoma at the age of 70.¹³ The risk of fallopian tube cancer is difficult to estimate,¹⁴ but also seems to be increased in BRCA mutation carriers.^{15–17}

“Loss of BRCA function in DNA damage repair is in itself not sufficient to confer uncontrolled cell growth resulting in cancer, but may lead to the accumulation of other genetic hits”

An inherited mutation in BRCA1 or BRCA2 is considered to be the first important “hit” in most hereditary ovarian and fallopian tube (further denoted “adnexal”) and breast carcinomas,^{18–20} leading to impairment of function of this important tumour suppressor gene when the wild-type allele is lost.²¹

Loss of BRCA function in DNA damage repair is in itself not sufficient to confer uncontrolled cell growth resulting in cancer,^{22–24} but may lead to the accumulation of other genetic hits, of which inactivation of other tumour suppressor genes are the main events that lead to the loss of cell cycle control

and ongoing proliferation. However, genetic studies on hereditary ovarian cancer have failed to elucidate a common tumour suppressor gene involved in hereditary adnexal carcinogenesis in addition to the inactivation of BRCA1. The search for relevant tumour suppressor genes includes the analysis of loss of heterozygosity (LOH) by the polymerase chain reaction (PCR) and comparative genomic hybridisation (CGH). Such approaches have revealed many chromosomal regions deleted in ovarian tumours.^{5 25 26} Chromosome 13 has warranted special attention, because high frequencies of LOH on chromosome 13 were seen in up to 50% of sporadic ovarian carcinomas.^{26 27} In addition, in a recent CGH study on hereditary ovarian cancer by our group, chromosomal loss on 13q was a frequent event.²⁸ Our present study was designed to investigate further the relevance of the high frequency of LOH on chromosome 13 in BRCA1 related adnexal carcinomas, with the hope of identifying regions that may contain putative tumour suppressor genes. To this end, we performed LOH PCR for seven highly polymorphic markers distributed over the long arm of chromosome 13 in a group of 36 BRCA1 related adnexal carcinomas.

MATERIALS AND METHODS

Patients

Two fallopian tube carcinomas (cases 901f and 902f) and 34 ovarian carcinomas from confirmed BRCA1 mutation carriers

Abbreviations: CGH, comparative genomic hybridisation; LOH, loss of heterozygosity; MSI, microsatellite instability; PCR, polymerase chain reaction; RB1, retinoblastoma gene

Table 1 Frequency of loss of heterozygosity (LOH), retention (R), microsatellite instability (MSI), and non-informative results because of homozygosity (X) or non-evaluable PCR (-) for seven microsatellites spanning chromosome 13q in 36 BRCA1 related ovarian and fallopian tube carcinomas. BRCA1 mutation and, if known, grade and FIGO stage are indicated

Patient	Marker							BRCA1 mutation	Grade	FIGO
	D13S175 (13q11)	D13S26R (13q12)	D13263 (13q14)	D13S312 (13q21)	D13S271 (13q22-31)	D13S71 (13q32)	D13S173 (13q32-4)			
1	R	X	LOH	R	-	R	X	3937insG	2	IV
2	R	LOH	X	X	X	R	X	2804delAA	3	IIIb
3	R	R	R	R	R	LOH	-	1240delC	3	IIIc
4	LOH	-	LOH	-	LOH	X	LOH	2804delAA	3	IV
5	LOH	X	MSI	-	R	X	R	2804delAA	3	IIIb
6	-	LOH	-	-	X	-	-	2457C→T	2	III
7	X	LOH	X	-	-	LOH	-	1410insT	2	III
8	X	R	LOH	LOH	LOH	X	X	IVS2+1G→T	2	IIIc
9	X	R	R	LOH	LOH	LOH	X	3875del4	2	IIIc
10	X	LOH	LOH	LOH	LOH	LOH	LOH	2576delC	2	IIIc
11	X	R	R	-	LOH	MSI	R	2388delG	1	IIIa
12	R	LOH	LOH	LOH	LOH	LOH	LOH	2080insA	3	IIIc
13	X	R	R	X	R	R	R	E730X	2	IV
14	MSI	MSI	LOH	LOH	LOH	LOH	LOH	G1788V	2	IIIc
15	-	-	-	-	-	MSI	X	2804delAA	3	IIc
17	LOH	-	LOH	-	-	X	LOH	2841G→T	2	IIIc
19	X	R	LOH	X	LOH	LOH	LOH	1410insT	2	IIIc
20	-	-	-	-	-	X	R	1136insA	1	
21	X	X	LOH	X	X	LOH	X	2575delC	3	IV
22	X	R	R	X	X	R	R	D173G	2	IIIa
23	R	R	R	LOH	R	LOH	R	IVS5-11T→G	2	IIIc
109	LOH	LOH	LOH	LOH	-	R	LOH	5382insC	2	III
125	X	-	X	-	X	-	X	T3053A	2	III
143	LOH	LOH	MSI	X	LOH	LOH	LOH	2190delA	3	III
145	R	X	LOH	R	X	X	X	2819delTT	2	Ia
153	X	R	X	R	X	R	X	3875del4	2	III
154	LOH	R	LOH	-	X	LOH	X	185delAG	2	
301	X	-	LOH	-	-	-	-	3450del4	2	
302	-	-	-	-	-	-	-	3121 delA	2	
304	LOH	-	-	-	-	-	-	Arg1835ter	3	
305	X	-	-	-	X	-	-	lys607ter	3	
306	X	-	-	-	-	-	-	5382insC	3	
307	R	-	LOH	-	-	-	-	3875del4	3	
308	R	-	X	-	-	R	-	4184del4	2	
901f	X	LOH	X	LOH	X	R	LOH	1410insT	2	
902f	LOH	LOH	R	X	X	LOH	X	2804delAA	3	IIc

were collected from three institutes (London, Toronto, and Amsterdam). BRCA1 mutations had been established before by various strategies including the protein truncation test and direct sequencing. FIGO stage distribution was: stage I, one case; stage II, two cases; stage III, 19 cases; and stage IV, four cases; the FIGO stage of 10 tumours was unknown. Archival, formalin fixed, paraffin wax embedded histological specimens were reviewed. Histopathological review confirmed serous cystadenocarcinoma in all cases. The grade distribution was as follows: two cases of grade I, 21 of grade II, and 13 of grade III. Table 1 summarises the data of the individual subjects.

DNA isolation

DNA was isolated as described previously.¹⁷ Briefly, normal and tumour tissue were isolated from areas of 3–6 mm² in 10 µm thick paraffin wax sections, guided by haematoxylin and eosin stained sections in which the tumour and the normal areas had been marked by the pathologist (PvD). A laser microdissection microscope was used when necessary (Leica, Rijswijk, the Netherlands). The isolated tissues from each subject were pooled and DNA was extracted in 50 µl of lysis buffer (100mM Tris/HCl (pH 8.8), 2mM EDTA, and 400 µg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany)). The samples were incubated overnight at 56°C, boiled for eight minutes to inactivate proteinase K, and stored at 4°C.

DNA amplification

The isolated DNA was amplified by PCR with primers for seven microsatellite markers on chromosome 13: D13S263 (13q14.1–2), D13S175 (13q11), D13S260 (13q12.3), D13S312 (13q21.1–3), D13S271 (13q22–31), D13S71 (13q32), and D13S173 (13q32–4). These markers were chosen because of more than 70% heterozygosity in the population. Moreover, the choice of the markers was restricted to amplification products of at most 200 bp because DNA isolated from paraffin wax blocks can be fragmented.

DNA was amplified in a final volume of 15 µl of PCR mix including dNTPs (dATP, dTTP, and dGTP, each at 200µM), dCTP at 50µM, dCTP-CY5 (Amersham Pharmacia Biotech, Buckinghamshire, UK) at 1µM, 0.67µM of each primer, and 0.1 U of Taq polymerase (Life Technologies). PCR was carried out in a thermal cycler PTC-100 (MJ Research, Watertown, Massachusetts, USA) under the following conditions: after an initial denaturation step of three minutes at 94°C, DNA was amplified through 35 rounds; each cycle consisting of a one minute denaturation step at 94°C, a one minute annealing step at 58°C (five cycles), 57°C (five cycles), and 55°C (25 cycles), and a one minute elongation step at 72°C.

The amplified PCR products were analysed using the Long-Read Towersystem V3.1 (Visible Genetics Inc, Toronto, Canada). The gels were loaded with CY5 labelled PCR products and run in accordance with the suppliers protocol. As a control, CEPH DNA was used to validate the expected length

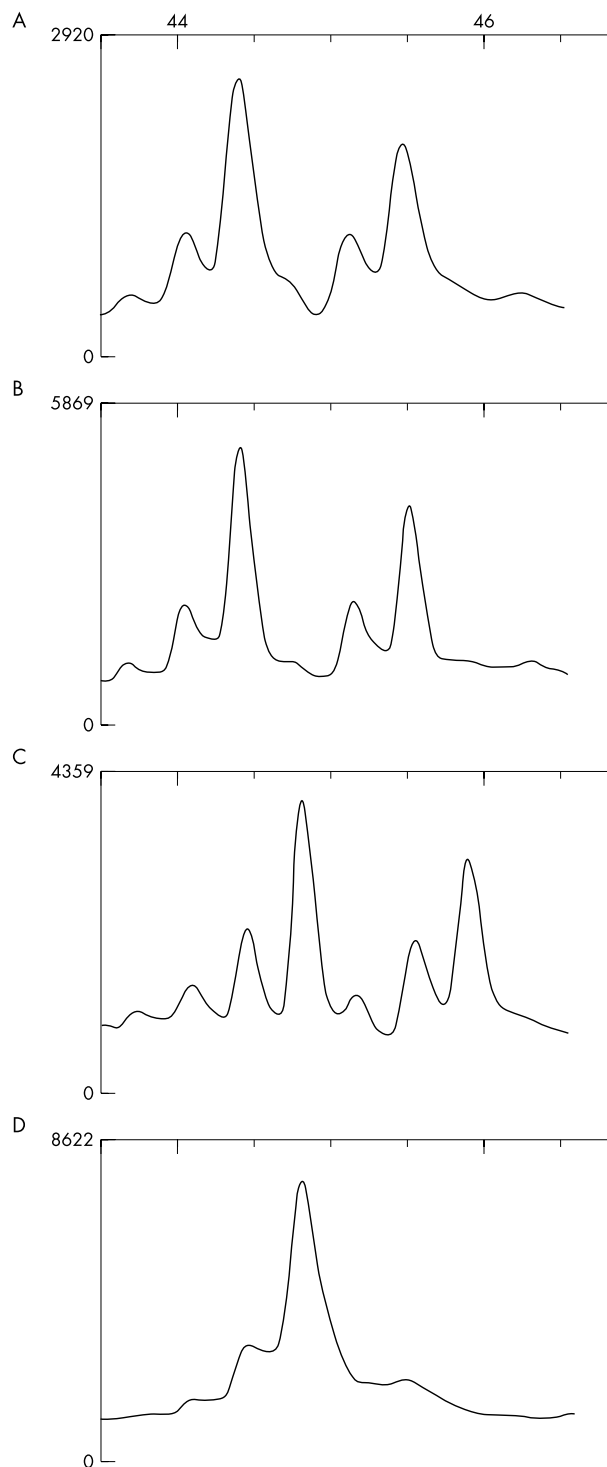


Figure 1 Computer representation of the polyacrylamide gel fragment analysis of CY5 labelled PCR products from patient 24 ((A) normal and (B) tumour cells) and patient 23 ((C) normal and (D) tumour cells). The alleles amplified from normal DNA with the D13S271 primer set are represented by two peaks showing heterozygosity in both cases (A and C). DNA obtained from tumour tissue of patient 24 showed both the alleles (B; retention), whereas in the tumour of patient 23 loss of heterozygosity was seen (D).

of the amplified products, which appeared, in all cases, to be within the expected size ranges.

As a control for baseline LOH in BRCA1 related carcinomas, LOH frequency on chromosome 15 was determined in the same subset of tumours as described above with markers

D15S1364, D15S118, D15S117, and D15S87, and the 99% confidence interval for this proportion was calculated.

Analysis of data

Results were read on computer print outs by at least two observers (AJ/JKG/RPZ/JMJP) until consensus was reached. In some cases, PCR and reading of the amplified products were repeated to improve the quality of the results. The area under the curve was calculated for both alleles in normal and tumour tissue, ignoring stutter bands. PCR products were classified into five categories, namely: retention (R), LOH, non-informative (X), microsatellite instability (MSI), and non-evaluable (-). Retention was defined as when the PCR products of both alleles recognised in the normal tissue were equally amplified, or differed by less than 50% (fig 1). A difference of at least a factor of two between the amount of DNA of such heterozygous alleles was designated as LOH, because even microdissected tumour material will be contaminated to some extent with normal cells. MSI represented a shift of the pattern, resulting from the loss or insertion of base pairs. If only one PCR product could be recognised in the normal tissue, because the individual was homozygous for the amplified sequence, the chosen marker was non-informative. Finally, cases were assigned non-evaluable when no PCR product from either tumour or normal tissue could be obtained. PCR amplification with poor results, obtained in a subset of the preparations, might be the result of suboptimal fixation and/or storage conditions. For some of the markers reliable results were obtained after repeated attempts. Lack of sufficient normal tissue in these sections hampered further investigations. MSI might be involved in tumorigenesis but because MSI provides no indication for the presence of a tumour suppressor gene, cases with MSI were excluded from the estimation of LOH frequencies.

RESULTS

Table 1 shows the LOH patterns over chromosome 13, the detected BRCA1 mutation, and grade (GOG) and FIGO stage if known for the 36 cases studied. In 10 cases, LOH patterns could be evaluated for at least five loci. Some of these patterns point to a deletion of the entire long arm of chromosome 13 (cases 10, 14, and 143) because LOH is present for all informative markers spread over the chromosome. In four cases (3, 9, 12, and 19) a variable number of q terminal adjacent markers are lost, pointing to a partial deletion of the long arm of chromosome 13, because at least one of the proximal markers showed retention. In only one case was retention of all informative markers seen (case 13). However, in many cases, the LOH pattern seemed to be fairly random, with retained parts in between lost parts and vice versa, which can be explained only by multiple complex chromosomal breaks and rearrangements (for example, 23 and 109f), a pattern which is also seen in cases where less than five markers were informative (1, 2, 11, 145, and 154).

Six PCR products in five cases showed MSI, two for 13q14 and 13q32, and one each for 13q11 and 13q12.

Table 2 summarises the LOH data of the seven markers on chromosome 13. Overall, LOH frequencies for the markers investigated were high at 59%. LOH was most frequent (69%) at 13q22-31, closely followed by 13q14 and 13q21 (67%), 13q32, and 13q32-4 (60%). The frequencies of LOH were lowest at 13q11 (50%) and 13q12 (47%). Figure 1 shows a computer representation of 13q22-31 PCR fragment analyses of tumours showing retention in one case and LOH in the other. We found no differences in LOH frequencies between tumours of different grades, or associations between LOH frequencies and type of BRCA mutations (data not shown). LOH for the four markers on chromosome 15 was on average 20% (99% confidence interval, 4.6% to 47%).

Table 2 Frequency of loss of heterozygosity (LOH), retention (R), microsatellite instability (MSI), and non-informative results because of homozygosity (X) or non-evaluable PCR (-) for seven microsatellites spanning chromosome 13q in 36 BRCA1 related ovarian and fallopian tube carcinomas

Marker locus	D13S175 13q11	D13S260 13q12.3	D13S263 13q14	D13S312 13q21	D13S271 13q22-31	D13S71 13q32	D13S173 13q32-4	Total
LOH	8	9	14	8	9	12	9	69
R	8	10	7	4	4	8	6	47
MSI	1	1	2	0	0	2	0	6
X	15	4	6	7	11	6	11	60
-	4	12	7	17	12	8	10	70
% LOH	50%	47%	67%	67%	69%	60%	60%	59%

DISCUSSION

In the 36 BRCA1 related adnexal carcinomas studied we found high ($\geq 50\%$) LOH frequencies on chromosome 13 for the markers D13S175, D13S263, D13S312, D13S271, D13S71, and D13S173. This implies the presence of putative tumour suppressor genes at the loci 13q11, 13q14.1-2, 13q21.1-3, 13q22-31, 13q32, and 13q32-4, which may be involved in the pathogenesis of the hereditary form of adnexal adenocarcinoma.

Because there can be much "background" LOH in advanced tumours like adnexal cancers,²⁹ especially in the case of defective DNA repair genes such as BRCA1 or BRCA2 as seen in breast cancer,³⁰ one has to set a threshold to define a locus as harbouring a putative tumour suppressor gene. The baseline LOH value in BRCA1 related tumours can be read from the frequency of LOH on chromosome 15, which can be assumed to have a similar probability to be broken and (mis)repaired because it is similar in size to chromosome 13. Because this frequency is 20%, we decided to consider that an LOH frequency $\geq 50\%$ indicates the presence of putative tumour suppressor genes because this threshold is outside the 99% confidence interval of this proportion, and is therefore expected to indicate non-random chromosomal deletions.

"Our results suggest that different genes on chromosome 13 can be involved in the early carcinogenesis of BRCA1 related adnexal cancer"

LOH on chromosome 13 was previously also suggested to play a role in the carcinogenesis of these tumours because it was found to be relatively common in advanced sporadic ovarian carcinomas (up to 50%).^{25 27 29 31 32} Three LOH studies^{26 27 33} have shown a common region on chromosome 13—13q33-4—with a high frequency of LOH in high grade tumours. Because we found a frequency of 60% LOH in this region, it seems plausible that the presumed role of chromosome 13 in the progression of sporadic ovarian cancer holds also for the hereditary form. Cytogenetic and CGH analyses have shown that aberrations in ovarian carcinomas mostly include partial deletions or monosomies,^{34 35} so we assumed that the observed LOH represents a real loss of loci on chromosome 13. Therefore, the LOH on chromosome 13 is indicative of the presence of tumour suppressor genes. There are several candidate genes at the loci that are suspected to harbour tumour suppressor genes. Locus 13q14 harbours the retinoblastoma gene (RB1),³⁶⁻³⁸ but different studies have shown that the expression of RB1 is retained in most primary ovarian carcinomas,^{27 39 40} indicating that another yet unknown tumour suppressor gene near to RB1 may be involved. In non-BRCA1/BRCA2 related breast cancer families, CGH and linkage analysis revealed strong evidence for the presence of a tumour suppressor gene on 13q21-2.³⁰ Our observation of a high frequency of LOH at 13q21.1-3 in BRCA1 related adnexal cancer supports the presence of such a tumour suppressor

gene on this narrowed chromosomal region, involved in both hereditary breast and adnexal cancer. Other candidate genes localised to 13q21-2 are protocadherin 9, the serine/threonine protein kinase EMK, the human homologue of the drosophila dachshund gene, and ubiquitin C-terminal esterase L3.³⁰ No other genes in close relation with breast or ovarian cancer were found on 13q11, 13q21, or 13q22-3.

An interesting gene localised to 13q32-34 is involved in the excision repair system, which is impaired in xeroderma pigmentosum complementation group G (Cockayne syndrome) (www.gdb.org/gdbreports >2001).

Kerangueven and colleagues suggested that after impairment of BRCA1 function the next obligatory step in tumorigenesis is the inactivation of one of a limited repertoire of tumour suppressor genes, rather than one particular tumour suppressor gene that initiates a fixed cascade of events.⁴¹ This option has gained acceptance with respect to breast carcinomas^{22 23 41 42} and probably holds true for BRCA1 related adnexal carcinomas also, because we and other groups failed to find evidence of the involvement of one common tumour suppressor gene in hereditary ovarian carcinomas.

Indeed, our results suggest that different genes on chromosome 13 can be involved in the early carcinogenesis of BRCA1 related adnexal cancer. Additional chromosome 13 markers may narrow these regions to localise the genes of interest more precisely. However, because we also found a high frequency of breaks, particularly in chromosome 13, tiny deletions or small retained chromosome fragments may be missed by the technique used. Even an increased number of markers cannot exclude these possibilities in tumours with a high number of chromosomal breaks. In these cases, only the demonstration of the presence of genes themselves will be reliable. If one gene of several optional suppressor genes is required early in carcinogenesis, the subsequent hits may vary also, so that various gene expression profiles can be expected in BRCA1 related adnexal tumours. Hedenfalk *et al* showed different molecular pathways in BRCA1 related, BRCA2 related, and sporadic breast cancer using gene expression profiles.⁴³ It would be interesting to see whether relatively small differences in gene expression, owing to the impairment of different tumour suppressor genes and subsequent genetic aberrations, can be noted from microarray data within the group of BRCA1 related adnexal tumours. In future studies, we plan to apply CGH-array to narrow down the chromosomal regions of loss, in addition to microarray expression analysis to study gene expression.⁴⁴ In the future the analysis of microarrays may lead to the identification of candidate genes involved in the early pathogenesis of BRCA1 related adnexal cancer.

There were no correlations between LOH or MSI and grade or stage. However, these data need to be interpreted with some care, because there were few grade I cases, and stage was usually 3 or 4 and lacking in several cases.

In conclusion, there are several loci on chromosome 13 (13q11, 13q14.1-2, 13q21.1-3 13q22-31, 13q32, and 13q32-4)

Take home messages

- High frequencies of loss of heterozygosity (LOH) were found on several loci on chromosome 13q, namely: 13q11, 13q14, 13q21, 13q22–31, 13q32, and 13q32–4
- Thus, putative tumour suppressor genes on the long arm of chromosome 13 may play a role in the pathogenesis of BRCA1 related ovarian and fallopian tube cancer
- LOH patterns appeared to be independent of the type of BRCA1 mutation, stage, and grade
- In the future, microarray analysis may lead to the identification of candidate genes involved early in the pathogenesis of BRCA1 related adnexal cancer

that show high frequencies of LOH in BRCA1 related adnexal cancer, and may therefore harbour putative tumour suppressor genes involved in the carcinogenesis of this particular type of hereditary cancer.

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