Published in final edited form as:

Cancer Genet Cytogenet. 2008 May ; 183(1): 41–48. doi:10.1016/j.cancergencyto.2008.02.00.

Genomic structure of chromosome 17 deletions in *BRCA1* associated ovarian cancers

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Abstract

The aim of this study was to determine the genomic structure of the deletions on chromosome 17 in ovarian carcinomas from women with inherited *BRCA1* mutations. Normal and tumor DNA from 14 ovarian tumors associated with inherited *BRCA1* mutations were extracted and tested for loss of heterozygosity (LOH) at microsatellite markers along chromosome 17. Finer mapping using more microsatellite markers and single nucleotide polymorphisms (SNPs) helped further define the LOH margins. The genomic repeated elements within the LOH breakpoint regions were identified using the University of California Santa Cruz Genome Database and the frequencies were compared to regions of equal GC percentages across the genome. Of the 14 ovarian tumors, 12 showed LOH of the entire chromosome 17. The other two tumors lost the distal end of the 17q arm. The breakpoints of these two tumors occurred in regions with significantly high frequencies of SINE repeating elements, specifically *Alu* elements. Ovarian tumors of high grade and stage have large regions of LOH along chromosome 17, with most tumors showing loss of the entire chromosome. In those tumors with retention of part of chromosome 17, LOH margins suggest that a high *Alu* content may have a role in the deletions.

Introduction

Ovarian cancer is the eighth most common cancer in women and the leading cause of death from gynecological malignancy in the Western world [1]. Between 10 and 15% of ovarian carcinomas are inherited; when diagnosed before the age of 50 years, approximately 80% of inherited ovarian carcinomas is due to mutations in the *BRCA1* gene [2]. Women with germline mutations in *BRCA1* have a cumulative risk of ovarian cancer of 40–52% by the time they reach the age of 70 [3].

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According to Knudson's hypothesis [4], two mutations are needed for cancer development: the first mutation ("hit") may occur either as an inherited mutation or a somatic mutation, whereas the second mutation ("hit") only occurs somatically. When the first hit is not inherited, both mutations occur in somatic cells; these 'sporadic' cancers happen later in life than inherited cancers due to the chance of having two mutations occur in the same gene, when each mutation occurs at a rate of approximately 2×10^{-7} per year [4]. The women who inherit mutations in *BRCA1* already have the first "hit" in ovarian carcinogenesis. The second hit, most commonly, is a deletion of the wild-type *BRCA1* allele shown as loss of heterozygosity (LOH), leading to no functional protein product of the gene.

Many chromosomal regions are altered in ovarian cancer, with the average number of alterations (chromosome gains and losses) being 9.17 ± 4.25 per tumor in *BRCA1* mutation carriers [5]. In comparative genomic hybridization (CGH) studies, ovarian cancers from *BRCA1* mutation carriers have been determined to have both regions of amplifications and deletions. The most common genomic regions of copy number gains include chromosomes 1, 2q, 3q, 5p, 6p, 7q, 8q, 11q, 19q, and 20, while the most common chromosome copy number losses occur on 3p, 4, 5q, 6q, 8p, 9, 12q, 13q, 17, 18q, 19, and X [5–7]. Loss of the entire chromosome 17, as revealed by LOH of all polymorphic markers, is quite frequent in ovarian cancer [8–16]. The loss of the entire chromosome could be due to both arms of chromosome 17 containing genes implicated in ovarian cancer: 17q21 contains *BRCA1*, 17p13 contains *TP53*, and other regions contain potential tumor suppressor genes [17 and references within].

The *BRCA1* locus is densely packed with repetitive elements, including 138 individual *Alu* elements that comprise 41.5% of the genomic sequence [18]; other repeats comprise 4.8% of the genomic sequence. *Alu* repetitive elements are associated with areas of high gene density and localize to R (reverse) bands of metaphase chromosomes [19], which are regions involved in homologous and nonhomologous chromosomal exchange [20]. It has been suggested that high densities of repetitive DNA elements could be frequent sites for homologous recombinations; thus, *Alu* sequences can serve as substrates for recombination following double strand breaks. If unequal crossing-over occurs between *Alu* elements intrachromosomally, deletions or duplications can result. If recombination occurs interchromosomally, chromosomal rearrangements such as translocations can result.

There are eleven subfamilies of *Alu* elements in the *BRCA1* gene that differ in sequence and in length [18], and yet these subfamilies can recombine with each other. The density and distribution of *Alu* elements in *BRCA1* suggests that this locus is particularly susceptible to suffering *Alu*-mediated loss of chromosomal material. *Alu*-mediated illegitimate recombination may lead to both germline and somatic mutation at the *BRCA1* locus. In some high-risk breast cancer families, complex germline rearrangements of *BRCA1* involving *Alu* sequences are associated with inherited breast and ovarian cancer [21–22]. Identification of these germline mutations and localization of their breakpoints support a role for *Alu*-mediated illegitimate homologous recombination as the mechanism responsible for chromosomal rearrangements [23].

The hypothesis of this study is that loss of heterozygosity could be due to rearrangements in repeat-rich genomic regions, similar to the rearrangements at *Alu* repeats in *BRCA1*. We wanted to delineate the exact LOH margins of the region deleted around *BRCA1* in ovarian cancer patients with germline *BRCA1* mutations. Studies in the past have used LOH studies to define genomic regions that may contain tumor suppressor genes, but have not actually looked at the genomic structure of these LOH borders. Perhaps Knudson's 'second hit' is occurring at these repeat sequences in addition to those rearrangements that occur in germline *BRCA1* mutations. Here, we performed a detailed study of LOH of chromosome 17 in fourteen ovarian cancers from patients with *BRCA1* mutations.

Methods and Materials

Patient selection

Patients with ovarian carcinoma were identified through the breast and ovarian cancer family studies performed by our lab or from the Gynecologic Oncologic Tissue Bank. Only patients from whom both tumor and normal tissue were available were included in the study. Blood and tissue were collected as approved by the Human Subjects Committee of the University of Washington Institutional Review Board. Thirteen patients, including one patient who had both an original ovarian tumor as well as a recurrent ovarian tumor, were determined to have *BRCA1* mutations through sequencing. The *BRCA1* mutation, histology, International Federation of Gynecologists and Oncologists (FIGO) staging, and cancer grade of each tumor used in this study are presented in Table 1.

DNA extraction

Normal DNA was obtained from blood draws for all but sample number 29502; these DNA samples were extracted from Epstein-Barr virus-immortalized lymphoblast cell lines using the Puregene DNA extraction system (Gentra). For the 29502 sample, normal DNA had to be obtained using normal tissue embedded into a paraffin block. For paraffin-embedded tissues, haematoxylin-eosin-stained sections were reviewed to find regions of tumor DNA (or normal DNA for sample 29502). The paraffin block or section of a block had to have at least 70% tumor cells in order to be considered the 'tumor region'. DNA was isolated from paraffin blocks using the QIAamp DNA Mini Kit protocol: isolation of genomic DNA from paraffin-embedded tissue followed by the tissue protocol (QIAGEN Handbook, 1999) or the Picopure™ DNA Extraction Kit (Arcturus Bioscience).

Markers

Microsatellite markers on chromosome 17 were chosen using the University of Santa Cruz Genome Database (www.ucsc.browser.com), where the primer sequences may be obtained. Markers with high heterozygosity, high copy number, and short length were preferentially chosen. Some markers were chosen not off of the STS Marker tract but off of the Perfect Microsatellite (July 2003) tract, and were labeled with their repeat unit followed by their chromosome location in megabases. Some SNPs were also chosen when performing the fine delineation of breakpoints. These SNPs were listed on the browser to have high heterozygosity percentages.

PCR and LOH detection

The isolated normal and tumor DNA samples were amplified by PCR for each set of marker primers. PCR was performed using a 25µl volume containing 100ng DNA, 10pmole each primer, 200µM of dATP, dTTP, and dGTP, 10µM dCTP, 1X PCR Buffer (Invitrogen), 1.5mM MgCl₂, 1 unit Taq polymerase, and 500µCi [α -³²P] dCTP. The PCR conditions were an initial denaturation step of four minutes at 94°C, followed by 35 cycles of 94°C for 30 seconds, 50°C to 62°C (depending upon primer annealing temperatures) for 30 seconds, and 72°C for 30 seconds, and then an elongation step of 2 minutes at 72°C. Amplified products were diluted 1:1 with loading buffer (98% formamide, 10mM EDTA, 0.02% bromophenol blue, 0.02% xylene cyanol FF), denatured at 95°C for 3 minutes, and then immediately placed on ice. The products were loaded onto a 6% polyacrylamide/urea gel and electrophoresed. The gels were dried under vacuum at 80–90°C and then exposed to radiographic paper. To determine if the tumor DNA had LOH at a certain marker, the normal DNA was first investigated for heterozygosity at the marker site; if the marker was informative, tumor DNA and normal DNA were amplified and evaluated side by side. LOH was identified by visual determination of a relative reduction of at least 50% of one of the alleles compared to the other allele, in tumor

compared to paired normal DNA. Because of the presence of small amounts of contaminating normal cells in tumor samples (<30%), the reduction in intensity of a deleted allele might have been less than 100%.

For SNP alleles, PCR reactions were carried out using non-radioactive dNTPs and then purified using Sephadryl 300 to remove unincorporated dNTPs and primers. Sequencing reactions consisted of 10µl including ~100ng PCR DNA, 3.2pmole primer, 1X Sequencing buffer, and 2µl BigDye® Terminator (v3.1 cycle) reaction mix. Sequencing reactions amplified with an initial denaturation step of four minutes at 94°C, followed by 35 cycles of 94°C for 10 seconds, 50°C for 5 seconds, and 72°C for four minutes, and then an elongation step of 2 minutes at 72°C. To each sequencing reaction, 5µl of 125mM EDTA and 65µl of 100% ethanol was added. After a precipitation time of 15 minutes to 16 hours, sequencing reactions were centrifuged at 3820rpm for 30 minutes. The supernatant was then discarded and 100ml of 70% ethanol was added. The reactions were then centrifuged again at 3820rpm for 20 minutes. The supernatant was again discarded, the reactions dried, and then 15µl HiDi™ formamide (Applied Biosystems) added, to sequence on the ABI 3100 sequencer. Sequences were analyzed using either Sequencing Analysis (version 3.3) or Sequencher™ (version 4.2). Normal DNA was first sequenced to look for heterozygosity at the SNP, and then tumor DNA was sequenced to look for loss of an allele (or a reduction of one of the alleles by more than 50%) at that SNP.

Repeat detection and statistical analysis

DNA between the two markers or SNPs defining the LOH border was determined using the UCSC Genome Database (www.ucsc.browser.com). The Repeating Elements by RepeatMasker Track was put in full. Each element was clicked on, and the class, family, and genomic size recorded. Lengths of elements of the same class and family were added together to determine total length and percentage of repeat elements within the LOH breakpoint regions. The GC percentage of the entire DNA region was calculated using the web program located at http://www.genomicsplace.com/gc_calc.html. Using the GC percentages of each region, the expected percentage of each repeat type was derived [24]. Observed versus expected frequencies of *Alu* and other repeats in genomic regions of interest were compared using χ^2 . All p-values were two-tailed.

Results

LOH analysis along chromosome 17

Fourteen ovarian cancers with *BRCA1* mutations were studied. All of these cancers were of high stage and grade. Using both normal and tumor DNA, these cancers were analyzed for loss of heterozygosity at markers along chromosome 17 (Figure 1). Markers within *BRCA1* (D17S932, D17S855, D17S1322, and D17S1323) were first analyzed to confirm loss of the wild-type allele. Thirteen of the fourteen tumors had loss of heterozygosity at this gene, identified as LOH in at least one of the four markers within *BRCA1*. Only one tumor, 29502, was not informative at all four markers.

In order to determine if it was just the region around *BRCA1* that was lost in all tumors, markers close to the gene in both the proximal and distal directions were next tested. For tumor 29502 (which did not have informative markers within *BRCA1*), the closest proximal and distal markers to *BRCA1*, D17S800 and D17S633 respectively, were determined to have loss of heterozygosity, indicating that the *BRCA1* gene may have indeed undergone loss of heterozygosity. Distally from *BRCA1*, all tumors appeared to have lost the entire end of the 17q arm, as revealed by LOH through the end of the chromosome. Proximally from *BRCA1* to the centromere, twelve tumors showed LOH, suggesting that these twelve tumors lost the entire 17q arm. Two tumors, 6403 and 29502, retained heterozygosity somewhere between

D17S798 and *BRCA1* for tumor 6403, and somewhere between D17S1833 and D17S800 for tumor 29502. These regions could not be more finely determined with the initial set of microsatellite markers due to uninformative markers.

Four sites along the chromosome 17p arm were chosen to determine if tumors had undergone loss of heterozygosity of the entire arm. These sites included the distal end of 17p (markers D17S1866, (GT)0.08, and D17S849), markers around or within the *TP53* gene ((AAT)7.5, (TATC)7.5, and D17S1353), markers located in the middle of the chromosomal arm (D17S520 and (GAA)11.1)), and markers near the centromere on 17p (D17S793, D17S2187, and D17S1871). The twelve tumors that lost the entire chromosome 17q arm again showed LOH at markers throughout the 17p arm, suggesting that they had lost the entire chromosome. The two tumors (6403 and 29502) that retained part of the 17q arm also retained at least part of the 17p arm since heterozygosity was retained at the 17p centromeric markers.

Finer delineation of retained regions of chromosome 17

Microsatellite markers and SNPs were chosen between the previously defined borders (Figure 1) of LOH and retention of heterozygosity for tumors 6403 and 29502. For tumor 6403, the breakpoint occurred between markers D17S798 and D17S855, a 10.1MB region. For tumor 29502, the breakpoint occurred between markers D17S1833 and D17S800, a region of 5.2MB. Figures 2 and 3 show the originally defined borders as well as the more finely derived breakpoints. Multiple SNPs and microsatellites were uninformative within both of these regions and are not shown. The LOH border of tumor 6403 was within a 58.4 kB region between markers D17S1299 and SNP rs1468259, while the LOH border of tumor 29502 was within a 23.5 kB region between markers D17S18676 and SNP rs9894479.

These regions have numerous repetitive elements, as detailed in Table 2. For comparison, the average percent of repetitive units in genomic segments [24] are also shown in Table 1. The region in tumor 6403 contains 34% repetitive elements, within a region that is 40.0% GC rich. The most common repetitive elements in this region are SINEs, specifically *Alu*. The region in tumor 29502 contains 40% repetitive elements, in a region that is also 40% GC-rich. More than half of the repeats within this region are SINEs, the majority of which are *Alu* elements. Compared to other genomic regions with similar GC content [24], the high frequencies of *Alu* and MIR elements and low frequencies of all other repetitive elements are highly significant with p-values less than 10^{-10} . The total distribution of repeat elements between the deleted regions of tumors 6403 and 29502 compared to other genomic regions of similar GC content were highly significant (p-value < 10^{-30}).

Discussion

The goal of this study was to delineate the loss of heterozygosity borders on chromosome 17 surrounding the *BRCA1* gene in ovarian tumors from *BRCA1* mutation carriers. However, like many previous studies, many of the tumors used in this study were determined to have lost the entire chromosome. The loss of the entire 17q was not unexpected due to the high stage and grade of these cancers, but loss of the 17p arm was more surprising. Loss of the 17p arm was not correlated to mutations within the *TP53* gene since some tumors without identified mutations in this gene still had loss of the chromosome arm (*TP53* data not shown). Perhaps *TP53* mutations in these tumors were not identified, or perhaps the loss of the chromosomal arm was due to other factors.

Loss of the entire chromosome in twelve of the fourteen tumors did limit the scope of this study, since only two tumors in which to define the LOH borders remained. These two tumors were not distinguishable from the other twelve tumors; they did not have a different histology, occur at early or later ages, or have a different *BRCA1* mutation. Though these two tumors did

not lose the entire chromosome, they still lost a large part of it; they lost a large quantity of the 17q arm, beginning at a point proximal to *BRCA1* through the telomere of 17q. Hence, the tumors only had one LOH border. Using microsatellite markers and SNPs, the border was narrowed to a region of 58,394 basepairs in tumor 6403 and 23,460 basepairs in tumor 29502. Lack of informativity in numerous SNPs prevented further refining of each region.

Many repetitive elements were identified within each LOH breakpoint region. Within the 6403 region, repeats made up 34% of the sequence; within the 29502 region, repeats made up 40% of the region. Compared to a typical region of the genome that is 40% GC rich, both the 6403 and 29502 LOH borders were not as rich in total repetitive elements, but they did have a higher SINE percentage. Compared to the 10.0% *Alu* percentage in a typical genomic segment that is 40% GC rich, the 6403 region consisted of 12.7% *Alu* and the 29502 region consisted of 17.2% *Alu*. Also, the MIR percentages were 3.17% for the 6403 region and 4.34% for the 29502 region compared to the typical 2.2%. These increases in SINE repeats were significant with p-values less than 10^{-10} . Repeat-rich sequences are believed to promote recombination; for example, inherited genetic rearrangements in the *Alu*-rich *BRCA1* locus have been identified. Hence, perhaps the high percentage of *Alu* elements facilitates rearrangements or the loss of DNA revealed in loss of heterozygosity for these regions.

As stated before, the grade and stage of the tumors used were quite high, consistent with previous observations of *BRCA1*-associated ovarian cancer [25]. It is possible that with lower stage tumors, loss of heterozygosity borders would have been smaller, or at least not encompassing the entire chromosome, hence allowing the identification of the DNA architecture at LOH borders. Of even more potential in studying the questions asked in this report is using *in situ* ovarian cancers. In prophylactic salpingo-oophorectomies of women with *BRCA1* mutations, a small region of cancerous cells is sometimes identified. If studies could be performed on these low stage but high grade tumor samples, small regions of LOH may be identified. The finding of small LOH regions could suggest that the size of the loss of heterozygosity region increases with tumor progression. Even if these cancers have large LOH regions, one could suggest that larger LOH events are an early event in development of *BRCA1*-associated ovarian cancers.

Even though all of the ovarian tumors in this study showed loss of heterozygosity of the wild-type chromosome for at least some part of chromosome 17, these results do not definitely demonstrate a change in allele number. Loss of heterozygosity is a relative measurement and does not exclude the possibility of allelic gain as it cannot quantify the number of alleles; LOH can only reveal if a once heterozygous marker is no longer heterozygous. Often, through FISH (fluorescence *in situ* hybridization) or Q-PCR (quantitative PCR), the number of alleles at the LOH site is actually quantified to be two, with a duplication occurring of the mutant allele. For example, acute myeloid leukemia (AML) cells with terminal LOH (LOH that extended from a missing SNP to the telomere, similar to tumors 6403 and 29502 in this study) have been identified [26]. The allele composition in the LOH region in these terminal LOH tumors changed from one copy of each allele to two copies of the retained allele. Thus, this loss of heterozygosity resulted from a loss of a large region of the chromosome that was replaced by duplication of the corresponding region from the homologous chromosome (acquired partial uniparental disomy). This acquired partial uniparental disomy has been described in other cancers such as Wilms tumors [27–28], breast carcinomas [29], acute lymphoblastic leukemia [30], melanomas [31], in cancer predisposition syndromes [32], and in polycythemia vera [33].

In summary, twelve of the fourteen ovarian cancers of women carrying *BRCA1* mutations lost the entire chromosome 17, while the other two tumors lost only part of the 17q arm. The LOH

borders of the two tumors that retained partial heterozygosity of chromosome 17 were more rich in *Alu* sequences than a typical region of DNA with the same GC-richness.

Acknowledgements

The project described was supported by grant number F30 ES13069 for KMC from the National Institute of Environmental Health Sciences (NIEHS), NIH. Other research support was by NIH career development award K08CA096610 (EMS), NIH R01 ES013160 (MCK), and an award from the Yvonne Betson Trust (MCK and EMS). MCK is the Disney Foundation American Cancer Society Research Professor. The contents of this article are solely the responsibility of the authors and do not necessarily represent the official views of the NIH or other funding agencies.

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Figure 1. Loss of heterozygosity on chromosome 17 in fourteen ovarian cancers
 Microsatellite markers along chromosome 17 were tested in 14 inherited ovarian cancers. Location of each marker is noted on the left relative to its position on the chromosome. Loss of heterozygosity is noted by a black circle, retention of heterozygosity is noted as a white circle, and an uninformative marker is noted as a gray circle.

Figure 2. Finer delineation of LOH margin of ovarian cancer sample 6403
Microsatellite markers and SNPs between markers D17S798 and D17S855 were tested in tumor 6403 for loss of heterozygosity. Uninformative markers are not shown. Location of each marker is noted as well as its position on chromosome 17. Loss of heterozygosity is noted by a black circle, retention of heterozygosity is noted as a white circle, and an uninformative marker is noted as a gray circle. The region in which the breakpoint at chromosome 17 occurs is between D17S1299 and rs1468259, a region of 58,394 basepairs.

Figure 3. Finer delineation of LOH margin of ovarian cancer sample 29502

Microsatellite markers and SNPs between markers D17S1833 and D17S800 were tested in tumor 29502 for loss of heterozygosity. Uninformative markers are not shown. Location of each marker is noted as well as its position on chromosome 17. Loss of heterozygosity is noted by a black circle, retention of heterozygosity is noted as a white circle, and an uninformative marker is noted as a gray circle. The region in which the breakpoint on chromosome 17 occurs is between D17S1867 and rs9894479, a region of 23,460 basepairs.

Table 1
Characteristics of cancers used in LOH ovarian cancer study

Patient Number	Age at diagnosis	BRCA1 mutation ^a	Cancer site	Histology	Stage	Grade
6403	45	2411delAG	Ovary	Papillary serous	Unstaged	3
6404	50	2411delAG	Ovary	Papillary serous	Unstaged	3
8708	76	Δexon 20–22	Ovary	Endometrioid	Unstaged	not avail.
10201	54	2862delT	Ovary	Papillary serous	Unstaged	3
15303	38	Δexon 14–20	Ovary	Papillary serous	IIC	3
28201	39	185delAG	Ovary	Endometrioid	IIIC	3
28901	54	2594delC	Ovary	Papillary serous	IV	3
28901 rec ^b	55	2594delC	Ovary	Papillary serous	IV (rec)	3
29402	63	2800delAA	Ovary	Papillary serous	IIIC	3
29502	75	2800delAA	Ovary	Papillary serous	Unstaged	3
35001	76	L1407P;	Ovary	Papillary serous	IIIC	3
43501	41	185delAG	Ovary	Papillary serous	IIIC	3
50501	43	2798delGAAA	Ovary	Papillary serous	IIIC	3
96001	55	E1250X	Fallopian tube	Papillary serous	IIIC	3

^a Mutated basepairs are based upon the sequence from GenBank L78833; amino acid substitutions are based upon sequence AAC37594

^b Tumor samples are taken from the recurrent ovarian tumor

Table 2
Repetitive elements within the LOH breakpoints of tumors 6403 and 29502

Tumor: Breakpoint between (MB): Total basepairs: Repeat element	6403 36,248–36,307 58,394		29502 32,453–32,477 23,460		Expected, in region of 40% GC ^a	
	#	bp	%	#	bp	%
SINE: ^d						
Alu	30	7409	12.6	19	4035	17.20
MIR	14	1852	3.17	6	1019	4.34
LINE: ^b						
LINE1	12	3755	6.43	9	2137	9.11
LINE2	11	3013	5.16	2	232	0.99
L3/CR1	2	488	0.84	0	0	0
LTR elements: ^c	8	2051	3.5	2	647	2.76
DNA elements ^d	6	654	1.12	4	1237	5.27
Simple repeats ^e	5	219	0.38	0	0	0
Low complexity ^f	9	341	0.58	3	82	0.35
Total	97	19,782	33.88	45	9,289	40.02
						42.55

^aValues averaged between 38–40% and 40–42% GC level [24]

^bSINEs (short interspersed nuclear elements) include Alu elements and MIR (mammalian-wide interspersed repeat) elements

^cLINEs (long interspersed nuclear elements) include LINE1, LINE2, and L3/CR1 elements

^dLTR (long terminal repeat element) include MAl.Rs (mammalian LTR-retrotransposons), ERV (endogenous retroviruses) classes I and II (class II not shown because neither LOH region contained these elements), and ERVL (foamy virus-like) elements

^eDNA elements include transposons in the *Ac/hobo* and *Tc/mariner* classes

^fSimple repeats include di-, tri-, and tetra-nucleotide repeats

^gLow complexity regions include AT-rich regions