[CANCER RESEARCH 63, 4588–4593, August 1, 2003]

Widespread Bimodal Intrachromosomal Genomic Instability in Sporadic Breast Cancers Associated with 13q Allelic Imbalance[1]

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ABSTRACT

Genomic instability is thought to underlie tumor progression in solid tumors, such as breast cancer. Although evidence that the hereditary breast cancer genes, *BRCA1* and *BRCA2*, are involved in DNA repair suggests that genomic instability plays an important role in hereditary breast tumorigenesis, genomic instability remains poorly characterized in sporadic breast cancers. Using a DNA fingerprinting technique, inter-(simple sequence repeat) PCR (inter-SSR PCR), the degree of genomic instability was quantified in 47 sporadic breast cancers compared with matched adjacent normal breast tissues. Almost all sporadic breast cancers show significant genomic instability by inter-SSR PCR. The distribution of this instability is bimodal; 57% of the tumors show fewer changes, whereas 43% show striking genomic alterations. Further analysis of two inter-SSR PCR tumor–normal differences revealed a genomic amplification and probable deletion. Thus, inter-SSR PCR can detect chromosomal breakage-related genomic alterations in most sporadic breast cancers. Genomic instability as detected by inter-SSR PCR is not correlated with aneuploidy, suggesting that this technique preferentially detects intrachromosomal alterations. Chromosomal instability in breast cancer can therefore be subdivided into at least two groups: (*a*) intrachromosomal and (*b*) gross chromosomal. Allelic imbalance at markers at the *13q13* and retinoblastoma loci (13q) and not at 17q loci was significantly associated with high levels of intrachromosomal instability, suggesting genes at *13q13* and retinoblastoma loci are either selectively targeted or involved in the genesis of genomic instability in sporadic breast cancers.

INTRODUCTION

Genomic instability is thought to be the driving force underlying tumorigenesis in solid tumors. Several different forms of genomic instability have been described: (*a*) CIN,[3] which refers to gross chromosomal rearrangements, such as translocations and duplications; (*b*) MIN, which refers to postreplicative errors best observed at microsatellites in the DNA; and (*c*) nucleotide instability, which refers to single nucleotide mutations (1). Like other solid tumors, breast cancer is characterized by a variety of multifarious alterations at tumor suppressor genes and oncogenes, as well as in noncoding DNA (2, 3). However, the importance of the role of genomic instability in breast tumorigenesis has not been fully appreciated until recently. With the discovery of the *BRCA1* and *BRCA2* genes and their implication in the etiology of hereditary breast cancer, attention has been focused on the role of genomic instability in breast tumorigenesis. This is because both hereditary genes are implicated in DNA repair; BRCA2 has been found to be necessary for the efficient repair of radiation-induced DNA double-strand breaks (4, 5), whereas BRCA1 is required for transcription-coupled repair of oxidative DNA damage (6). The majority of breast cancers, *i.e.*, sporadic breast cancers, usually show alterations typical of CIN and not MIN: (*a*) aneuploidy; (*b*) AI; (*c*) karyotypic alterations; and (*d*) genomic amplifications (2, 3, 7). The elucidation of mechanisms underlying CIN has been hampered in part by the difficulty in quantitating the various genomic alterations present in breast cancers. Allelotyping can provide a genome-wide measurement of AIs but is very labor intensive, whereas DNA ploidy analysis suffers from a relative lack of sensitivity. Chromosomal analysis by optical-based techniques, such as karyotyping and CGH (8), allows the detection of copy number changes in very large chromosomal segments and was not designed for quantitative comparisons. Finally, CGH microarrays (9, 10) provide more sensitive genomic surveys, which, however, require the availability of expensive technological platforms and sophisticated bioinformatics analysis.

We reported previously the quantitation of global genomic instability in colon tumors using a genomic fingerprinting technique, inter-SSR PCR (11). Inter-SSR PCR involves the amplification of anonymous DNA segments between closely adjacent microsatellites, $(CA)_n$ repeats. As there are $\geq$50,000 $(CA)_n$ repeats scattered randomly throughout the human genome, such PCR reactions yield a multitude of products, in turn allowing the widespread representational scanning of the genome (11). Inter-SSR PCR can detect chromosomal breakage-related genomic alterations, such as small and large deletions. Importantly, this technique also allows cloning of the tumor-specific differences. Using inter-SSR PCR, 47 sporadic breast cancers were evaluated for genomic instability. We present data regarding the nature of some of the alterations detected by this technique. To begin to assess the role of BRCA1 and BRCA2 in genomic instability in sporadic breast cancers, we then determined whether AI at 17q and 13q loci surrounding these two genes was correlated with the observed genomic instability. We found that genomic instability is a widespread phenomenon in sporadic breast cancers and that the distribution of this instability is bimodal: 57% of breast cancers had low levels of instability, whereas 43% had high levels. Genomic instability as detected by inter-SSR PCR is distinct from aneuploidy, suggesting that it detects amplifications and deletions on an intrachromosomal scale. Interestingly, AI at the 13q loci was associated with high levels of instability, suggesting a possible role for BRCA2 or a gene in its vicinity in the genesis of a large subgroup of sporadic breast cancers.

MATERIALS AND METHODS

Subjects, Tissues, and DNA. Fifty-two consecutive patients at the Centre Hospitalier de l'Université de Montréal–Hotel-Dieu hospital with primary breast cancers signed informed consent for sampling of breast tumors and adjacent normal breast tissue from 1996 to 1998. Information regarding the following clinicopathological features was obtained from the charts: (*a*) age; (*b*) hormone receptor status; (*c*) histological and nuclear grade; (*d*) family history; and (*e*) presence of axillary lymphatic metastases (Table 1). Women with a family history of more than one relative with breast cancer were excluded from final analysis ($n = 5$). It was later found that one of these women was a *BRCA2* mutation carrier. One specimen was mistakenly obtained from a benign hemorrhagic breast cyst. Tissue was immediately frozen at the

Received 7/22/02; accepted 6/2/03.

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[1] Supported by the Linda Saab Foundation, Fondation de la recherche en chirurgie de Montréal, and the Montreal Breast Cancer Foundation (all to M. B.).

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[3] The abbreviations used are: CIN, chromosomal instability; inter-SSR PCR, inter-simple sequence repeat PCR; AI, allelic imbalance; RB, retinoblastoma; MIN, microsatellite instability; CGH, comparative genomic hybridization.

Table 1 *Clinicopathological features, genomic instability, allelic imbalance at 17q12-21, RB, and 13q13, and tumor ploidy*

									AI[f]						
									17q12-21		*RB*		*13q13*		
#DNA	Genomic instability index[a]	Age	ER[b]	PR[c]	Size (cm)	Histol. grade[d]	Nuclear grade	Axillary node status[e]	*17S588*	*17S250*	*13S153*	*13S218*	*13S260*	*13S171*	Ploidy
214	0	42	+	+	2.5	2	2	M	—	—	AI	—	ni	—	
90	1.25	56	+	+	0.9	2	2	M	AI	—	AI	—	AI	AI	Tetraploid
292	1.25	59	+	+	3.8	2	2	—	—	—	—	—	ni	—	Diploid
305	1.25	67	−	−	2.7	3	2	M	ni	—	—	ni	—	ni	
314	1.25	59	+	+	1.4	3	3	M	—	—	—	—	—	—	
15	2.5	61	+	+	2.8	3	2	—	AI	AI	AI	AI	AI	ni	
140	2.5	59	+	+	1.5	2	2	—	—	—	—	ni	—	—	
170	2.5	49	+	+	2.0	3	3	—	AI	AI	ni	—	AI	ni	
102	2.5	74	−	−	4.4	3	3	—	AI	AI	—	—	—	—	
116	2.5	56	−	−	1.5	3	2	—	—	—	ni	—	—	ni	
156	2.5	42	−	−	2.5	3	3	—	—	ni	—	—	—	ni	
164	2.5	55	+	−	1.5	2	3	—	—	—	—	ni	—	—	
166	2.5	67	+	+	3.0	3	2	—	ni	AI	—	ni	—	—	
277	2.5	57	+	+	2.5	3	3	M	ni	—	—	ni	ni	—	
288	2.5	47	+	+	5.0	3	2	M	—	—	—	ni	—	ni	
297	2.5	59	+	+	2.2	1	1	M	ni	—	—	AI	—	—	
172	3.75	37	+	+	2.0	2	2	—	ni	AI	—	AI	AI	AI	
78	3.75	63	+	+	4.0	2	2	—	—	—	—	—	—	ni	Diploid
114	3.75	60	+	+	2.2	2	2	M	AI	AI	ni	—	ni	—	Diploid
136	3.75	34	+	+	2.2	1	1	M	ni	ni	—	ni	—	ni	Diploid
152	3.75	50	−	−	4.5	3	2	M	ni	—	ni	—	—	ni	
387	3.75	71	−	−	1.5	3	3	—	ni	—	AI	—	—	ni	
100	5	46	−	−	1.5	3	3	M	ni	AI	—	ni	—	AI	Diploid
13	5	90	+	+	1.4	2	2	na	—	AI	—	AI	—	—	
26	5	79	+	−	6.0	1	1	—	—	—	—	—	—	—	Tetraploid
302	5	48	−	+	7.0	2	2	M	—	—	—	—	—	ni	
104	6.25	83	+	+	2.3	2	2	M	—	—	—	—	—	—	
338	7.5	85	+	+	2.5	1	2	na	ni	—	—	—	—	—	Diploid
385	7.5	81	+	+	4.5	3	2	—	—	AI	—	—	—	ni	Diploid
336	8.75	75	+	+	1.6	3	2	—	—	AI	AI	ni	AI	ni	
312	8.75	53	+	+	1.2	2	2	—	AI	—	—	ni	—	ni	Diploid
179	10	50	+	+	2.1	1	1	—	—	—	—	—	—	AI	Diploid
304	10	48	−	−	7.0	3	3	M	ni	ni	AI	ni	AI	AI	Aneuploid
183	10	75	+	+	1.5	1	1	M	ni	—	—	AI	—	ni	Diploid
3	11.25	66	−	−	5.0	3	3	—	ni	ni	AI	—	AI	AI	
154	11.25	65	+	−	3.0	3	2	M	AI	AI	AI	—	AI	AI	
185	11.25	48	+	+	2.9	3	2	M	—	—	AI	ni	ni	AI	
138	11.25	57	+	+	4.0		.	M	ni	—	—	—	—	—	Diploid
123	12.5	61	−	−	1.3	3	2	—	AI	ni	AI	—	AI	AI	Aneuploid
112	13.75	37	+	+	1.5	3	3	—	AI	AI	AI	ni	ni	ni	
118	13.75	71	+	+	1.5	3	1	—	—	ni	—	—	—	ni	Diploid
290	13.75	61	+	+	3.7	3	1	—	—	—	—	—	N	—	
92	15	80	+	−	2.9	2	2	—	—	AI	AI	AI	AI	AI	Tetraploid
287	15	75	−	−	2.2	3	2	—	ni	AI	ni	—	ni	AI	Aneuploid
106	16.25	54	na	na	2.0	3	3	—	AI	ni	AI	—	—	ni	Diploid
340	18.75	61	+	+	1.6	2	1	—	—	ni	—	—	—	—	
120	18.75	80	+	+	2.2	2	3	na	—	ni	AI	ni	AI	AI	
		% Allelic imbalance in informative tumors							30%	37%	33%	18%	28%	41%	
		% Informativity							70%	81%	89%	70%	85%	62%	

[a] Genomic instability index using inter-SSR PCR (number of alterations with both primers/80).
[b] Presence of estrogen receptor positivity by immunopathology (ER-ICA) as reported in the pathology report.
[c] Presence of progesterone receptor positivity by immunopathology (PRG-ICA) as reported in the pathology report.
[d] Histological and nuclear grades by Elston-Bloom-Richardson grading [1, 2, 3 (hi grade)].
[e] M, presence of axillary lymph node metastases; na, not available.
[f] ni, not informative; —, no allelic imbalance.

time of surgery and kept frozen at −86°C. Tumors and normal tissues were prepared for DNA extraction by using the "sandwich" technique; the frozen tissue samples were mounted for cryotome cutting. A first 6-μm section was made, followed by three to six sections of 50 μm, which were placed in 400 μl of buffer [10 mM Tris (pH 8) and 100 mM EDTA]. A second 6-μm section was then cut. Both 6-μm sections were verified for the percentage of tumor cells (>70% of tumor cells acceptable for tumors). Normal tissue sections were verified for the absence of tumor cells. The samples were digested with Proteinase K in the presence of 0.5% of SDS overnight at 37°C and then redigested with fresh addition of Proteinase K for 3 h at 50°C. This was followed by RNase digestion for 1 h at 37°C, phenol:chloroform:isoamyl alcohol extraction, and ethanol precipitation. Aliquots of final DNA preparations were run on agarose gels. Only samples without signs of DNA degradation were analyzed by inter-SSR PCR.

Inter-SSR PCR. Inter-SSR PCR was performed as described previously (11), with some modifications. $(CA)_8RG$ (R = a 50:50 mix of the purines adenine and guanine) and $(CA)_8RY$ (Y = a 50:50 mix of the pyrimidines cytosine and thymine) primers were synthesized in the Centre Hospitalier de l'Université de Montréal–Hôtel-Dieu biopolymer facility. Unlabeled $(CA)_8RG$ and $(CA)_8RY$ primers were used separately in two PCR reactions so as to produce two different fingerprints for each DNA sample. Amplification was carried out in a 20-μl reaction mixture consisting of 2 mM primer, 50 ng of genomic DNA, 0.26 mM deoxynucleoside triphosphate, 1 μCi of α-32P-labeled dCTP (3000 Ci/mmol; Amersham Canada, Oakville, Ontario, Canada), and 2 units of Taq polymerase (Life Technologies, Bethesda, MD) in PCR buffer [10 mM Tris-HCl (pH 9.0), 2% formamide, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, and 0.01% Triton X-100]. Cycling conditions of the Stratagene Robocycler were: (*a*) 5 min at 95°C followed by 30 cycles of 45 s at 95°C; (*b*) 1 min at 52°C and 2 min 15 s at 72°C; and (*c*) a final 7-min extension reaction at 72°C. Five microliters of reaction mixture were loaded onto a nondenaturing 8% polyacrylamide gel buffered with 1× Tris-borate EDTA. Electrophoresis was performed at 80 W for 22 min, followed by 1500 V/h for 4300 V/h. The gels were dried and placed on Kodak Biomax film for 2 days at room temperature. The assay was repeated six times for each tumor–normal tissue specimen pair to ensure reproducibility. Only reproducible differences (observed in at least four of six assays) in individual electrophoretic bands were

counted for each tumor–normal pair. The genomic instability index was the percentage of the total number of band changes with both primers divided by the average number of bands seen in normal breast tissue DNA with both primers ($n = 80$).

Analysis for AI. Microsatellite PCR was performed in a 20-μl volume containing 10 ng of normal or tumor DNA and 10 ng of each oligonucleotide primer, both of which were ^{32}P end-labeled. PCR primers were obtained from Research Genetics (Huntsville, AL) for the following loci: (*a*) *D17S588* (*17q21*, telomeric to *BRCA1*) and *D17S250* (*17q12*, centromeric to *BRCA1*) for 17q and *D13S260* (*13q13*, very closely, ~550 kb centromeric to *BRCA2*); and (*b*) *D13S171* (*13q13*, <200 kb telomeric to *BRCA2*), *D13S153* (*13q14*, intragenic *RB* marker), and *D13S218* (between the *BRCA2* and *RB* loci) for 13q. Samples were heated at 95°C for 3 min, then cycled for 1 min at 95°C, 1 min at annealing temperature, and 1 min at 72°C, followed by 3-min elongation at 72°C. Annealing temperature and number of cycles for each of the markers were as follows: (*a*) 61°C and 30 cycles for *D13S153*; (*b*) 55°C and 30 cycles for *D13S218* and *D17S250*; (*c*) 55°C and 28 cycles for *D13S260*; and (*d*) 55°C and 25 cycles for *D17S588*. PCR products were separated on 8% polyacrylamide denaturing gels, which were dried and placed on film for overnight exposure. AI was determined with the naked eye and, in ambiguous cases, by scanning densitometry of the autoradiographs. In the latter cases, tumors were scored as AI+ when a ≥50% difference in the relative allele intensity ratios was observed between tumor DNA and normal DNA.

Cloning and Analysis of Inter-SSR PCR Products. Bands of interest were cut out from inter-SSR PCR gels (both tumor and normal counterparts for each band), and DNA was eluted with 100 μl of water overnight at 4°C followed by ethanol precipitation. DNA fragments were cloned using a Taq cloning kit (Invitrogen, Inc.). For each band, ≤10 clones were selected, and the size of the cloned fragment was confirmed with restriction digestion with *Eco*R1. Further restriction digest of these clones revealed that some bands contained more than one different DNA fragment. Cloned DNA fragments were sequenced by BIO S&T (Montreal, Canada). Nested PCR primers for selected DNA fragments were synthesized by BIO S&T. The nested PCR primers described for the selected fragment [B6(T)5] shown in Fig. 3 were TGAGGAGTCCAAGGAACACA (forward) and CACCAGGGAAACACCACATT (reverse).

Southern analysis of tissue DNA was performed by *Eco*R1 overnight digestion, followed by agarose gel electrophoresis of the digestion products, and overnight transfer onto a nylon membrane. DNA loading was verified by assessment of the DNA smears and also by quantitation by absorbance before digestion. Unlabelled inter-SSR PCR products were also run on agarose gels for transfer. Hybridization with ^{32}P-random-labeled probes was performed using standard techniques.

Flow Cytometry. Flow cytometry was performed on 19 tumor samples in which enough tumor material was present after DNA extraction on a fluorescence-activated cell sorter machine at the Montreal Clinical Research Institute. Aneuploidy was defined as an extra peak not at G_1 or G_2. Predominantly tetraploid tumors were classified as aneuploid.

Statistical Analysis. Comparisons between the low and high instability groups for most values, including proportion of cases with AI in both groups, were performed using Fisher's exact test. The mean ages of the two groups were compared by Student's *t* test.

RESULTS

To quantify genomic instability in sporadic breast cancer, we analyzed 47 frozen breast tumors with a genome fingerprinting technique, inter-SSR PCR. All but one of the breast cancers showed detectable and reproducible alterations (Table 1). All differences were differences in band intensity and not appearances of new bands nor total loss of individual bands present in normal DNA (this latter type of change was not expected with a mixed tumor–normal cell population present in the "tumor" DNA; Fig. 1). There was an almost equal number of bands showing relative decreases as those showing relative increases in tumor DNA compared with normal DNA. As shown in Fig. 2, the distribution of instability indices is bimodal; a group of 27 tumors had relatively lower instability indices (median 2.5%), and a second group of 20 tumors had higher instability indices (median 12.5%). Both groups had similar numbers of increases as decreases in band intensity. There are no significant differences in clinicopathological features between the two groups (Table 2). Follow-up of these patients is too short (average 2 years) to reveal meaningful differences in survival. A preliminary Kaplan-Meier analysis did not reveal statistically significant differences between the two groups (data not

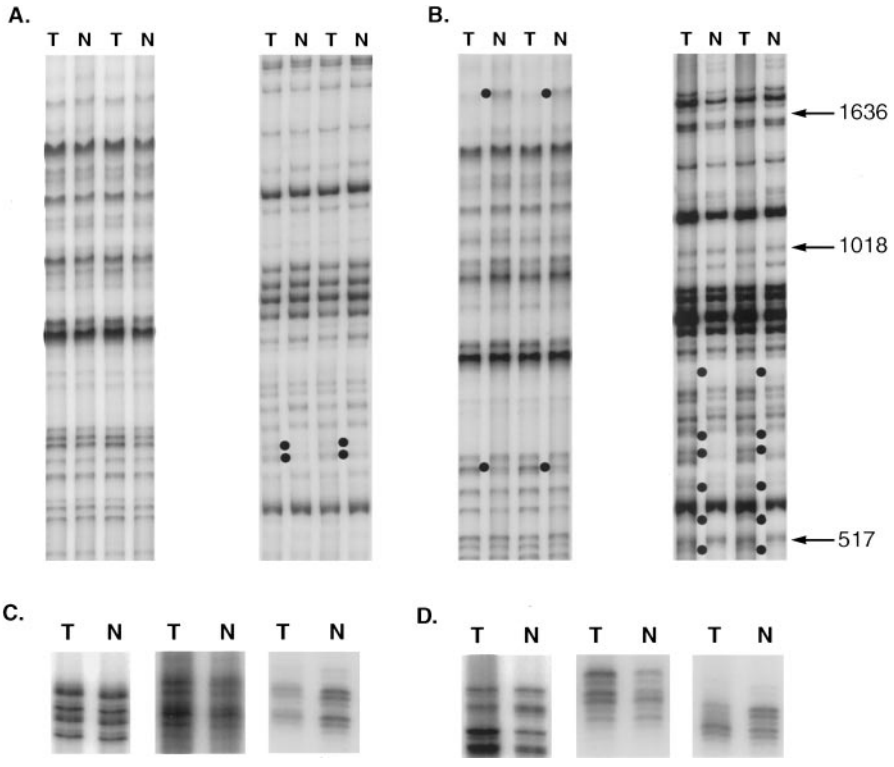

Fig. 1. *A*, fragments of gels of inter-SSR PCR analysis of breast tumor 102 (low instability group) in duplicate with $(CA)_8RY$ (*left*) and $(CA)_8RG$ (*right*) primers. *T*, tumor; *N*, normal. Altered bands are highlighted by dots between the tumor and corresponding normal lanes. *B*, same with breast tumor 304 (high instability group). Note that in this tumor, almost all of the differences are increases in intensity in the tumor DNA. DNA bp equivalents from 1-kb ladder at *right*. *C*, microsatellite PCR analysis of tumor 102 at the *BRCA2* markers (from *left* to *right*): *D13S171*, *D13S260*, and *D13S153*. Note no AI. *D*, microsatellite PCR analysis of tumor 304 at the same markers. Note AI at all three markers.

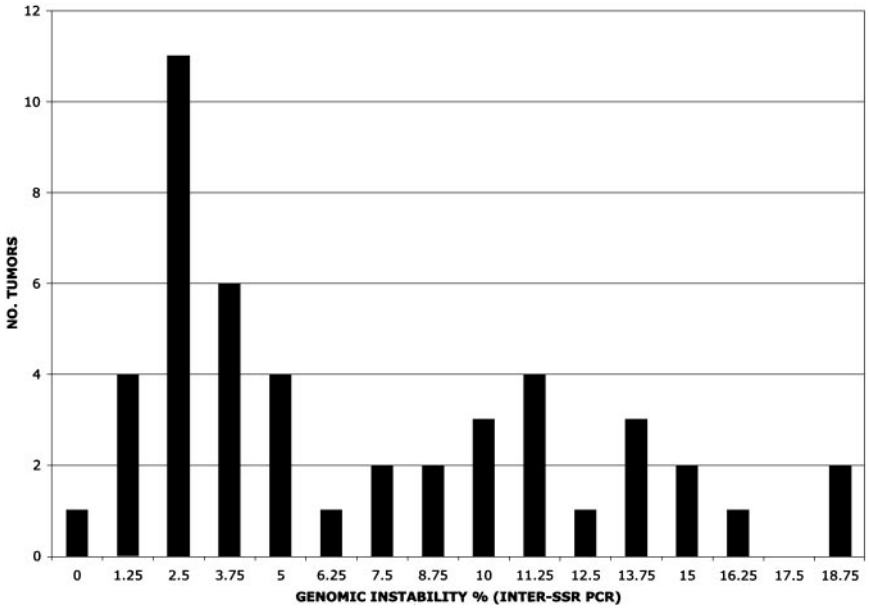

Fig. 2. Distribution of genomic instability indices in sporadic breast carcinomas. Inter-SSR PCR performed with two primers in 47 sporadic breast cancers. Note the bimodal distribution: low instability group with median at 2.5% instability and high instability group with median 12.5% instability.

Table 2 *Comparison of clinicopathologic features, allelic imbalance at 17q and 13q loci, and aneuploidy in low and high instability groups*

	Low instability[a] $n = 27$	High instability $n = 20$	Level of significance[b]
Mean age	55	58	ns
ER[c] +	19/27	15/19	ns
PR +	18/27	13/19	ns
Size (cm)	2.7	2.7	ns
High histological grade	13/27	12/19	ns
High nuclear grade	8/27	5/20	ns
Axillary lymph node +	14/26	5/18	ns
Family history +	3/25	5/18	ns
17q loci			
D17S588	5/18	5/14	ns
D17S250	8/25	6/13	ns
17q12-21[d]	9/27	9/18	ns
13q loci			
D13S260	4/23	7/17	$P = 0.09$
D13S171	3/16	9/13	$P = 0.006$
13q13[e]	5/27	10/19	$P = 0.008$
D13S218	4/18	2/12	ns
D13S153 (RB)	4/23	10/19	$P = 0.014$
13q13 and/or *RB*[f]	8/27	12/19	$P = 0.037$
Aneuploidy ($n = 19$)	2/7	4/12	ns

[a] Instability refers to genomic instability index as determined by inter-SSR PCR (percentage of altered bands in tumor DNA compared with normal breast tissue DNA).
[b] Tests for significance (Student's *t* test for mean age, Fisher's exact test for all other proportions) with $P < 0.05$ acceptable.
[c] ER, PR, proportion of tumors with positive estrogen or progesterone receptor by immunopathology (ER-ICA and PRG-ICA).
[d] Fraction of allelic imbalance at either or both 17q markers.
[e] Fraction of allelic imbalance at either or both *13q13* markers (*D13S260* and *D13S171*).
[f] Fraction of allelic imbalance at any of the *13q13* and *RB* markers (*D13S260*, *D13S171*, and *D13S153*).

shown). Inter-SSR PCR performed on the hemorrhagic breast cyst did not reveal any alterations.

To analyze the nature of the specific changes observed with inter-SSR PCR, we cloned and sequenced selected bands which were frequently altered in cancers. Nested PCR primers as well as DNA probes were then synthesized for these cloned DNA fragments and used to study the individual genomic changes one by one in specific tumor–normal pairs. Because several of the cloned inter-SSR PCR bands contained at least two different DNA fragments, the study of specific band differences sometimes entailed the analysis of several DNA fragments contained within that band. Results of the analysis of two inter-SSR PCR bands which frequently showed tumor–normal differences in breast cancers are presented here. One band showed an increase or decrease in intensity depending on the tumor specimen (Fig. 3). The other selected band showed an increase in intensity in breast tumor compared with normal DNA. The first case (Fig. 3*A*) corresponded to a single 1155-bp DNA fragment. Interestingly, we had also cloned the next top band on the inter-SSR PCR gel and found both to be identical except for a 12-bp insert located within a tet-

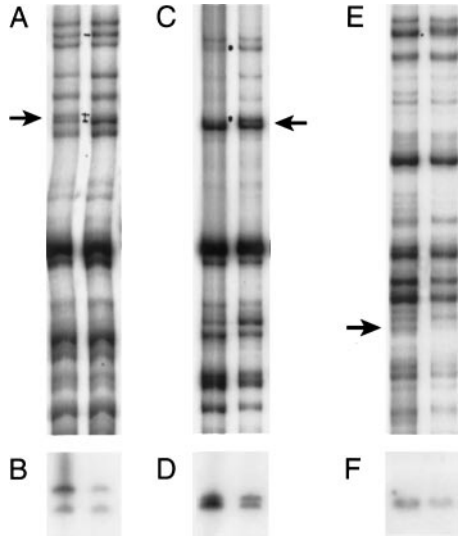

Fig. 3. AI and genomic amplification corresponding to inter-SSR PCR differences. *A*, inter-SSR PCR showing tumor-specific increase in band intensity (+), as well as relative decrease (−). *Arrow* points to the two adjacent bands of interest, the *top* of which is increased in tumor DNA, and the *bottom* is decreased. *B*, results of nested PCR primers based on the sequence from cloning of the top band indicated by the *arrows* in *A* and *C*. Note the top of the two nested PCR products, which corresponds by sequence to the inter-SSR PCR band of interest, showing increased intensity in tumor, analogous to increased intensity of the inter-SSR PCR product. Sequence analysis reveals the same tetranucleotide repeat (in capital letters below) number difference between the top and bottom band in both inter-SSR PCR and nested PCR primer; bottom band: cttaaTGAATGAATGAATGAATGGGctgac; top band: cttaaTGAATGAATGAATGAATGAATGGGTGGATGGATGGGctgac. *C*, inter-SSR PCR of another breast cancer showing tumor-specific decrease in top band intensity at the same band as in *A*. *D*, results of same nested primer PCR as in *B* showing this time a decrease in top band corresponding to a similar decrease observed in inter-SSR PCR in *C*. *E*, inter-SSR PCR showing a tumor-specific increase in indicated band. *F*, Southern blot analysis of *Eco*R1-digested tumor and normal DNA using a probe of one of the cloned DNA fragments from the indicated band showing relative amplification at this locus.

ranucleotide repeat in the top band. Nested PCR was performed for this fragment on tumor and normal DNA from two breast sample pairs, one which showed an increase and another sample which showed a decrease in the band in inter-SSR PCR. In both cases, two products differing by 12 bp were observed by nested PCR but with a different relative intensity ratio of the two. Suspecting that the intensity differences we observed were caused by AI at that locus in the tumor, we further analyzed the relative intensities of the two nested PCR products in tumor compared with normal DNA. In one tumor, a relative decrease of the top allele was observed that corresponded to the decrease in the analogous inter-SSR PCR band, whereas in the other, a relative increase in the top allele was observed, again matching the increase in intensity of the top inter-SSR PCR band (Fig. 3). Moreover, both DNA sequences are present in different normal breast DNA samples (data not shown), suggesting a polymorphism at this tetranucleotide repeat. This DNA product is homologous to a sequence present on the telomeric end of chromosome 10q, which has been shown to be a site for deletions in breast cancers. Thus, one of the inter-SSR PCR differences is caused by an AI, likely a deletion.

Three different candidate DNA fragments cloned from inter-SSR PCR of tumor DNA corresponded to the second band (which showed a tumor-specific increase in intensity in inter-SSR PCR). One of these fragments, measuring 789 bp long, was used to probe an inter-SSR PCR Southern blot and found to hybridize to both tumor and normal inter-SSR PCR products. To determine whether tumor-specific amplification of the clone could account for the tumor-specific intensity increase, Southern blot analysis using the original tumor and normal breast DNAs was performed. A 2-fold increase in band intensity of a unique DNA fragment in tumor DNA was found (Fig. 3). Although a relatively small difference, PCR amplification of such a difference could account for the greatly increased intensity observed on inter-SSR PCR fingerprinting. This DNA fragment maps to a pericentromeric region of chromosome 11, which has been shown to be amplified in breast cancers. Another of the three clones also maps to a telomeric region on chromosome 15q, where amplification has been detected by CGH in some breast cancers. Thus, this inter-SSR PCR difference may be caused by genomic amplification of at least one, if not both, of these DNA fragments.

To analyze the involvement of the 17q and 13q loci in these sporadic breast cancers, microsatellite markers adjacent to and on either side of the *BRCA1* and *BRCA2* loci were examined for AI. Two frequently heterozygous markers were used in each case to increase informativity. Representative results are shown in Fig. 1. None of the tumors showed evidence of MIN as defined by ≥40% of microsatellite markers showing typical novel alleles (13). Our overall rates of AI at informative cases, shown in Table 1, were comparable with those reported previously (14–18). Rates of AI at informative loci in the two instability groups are shown in Table 2. AI at loci on 17q was not significantly different in both groups of instability. Significant differences between the two instability groups were found for two loci on 13q: (*a*) *D13S171* (*13q13*) and (*b*) *D13S153* (*13q14* or *RB* locus). The most striking difference was found at the *13q13* locus; only 3 of 16 informative tumors with low instability showed AI at *D13S171* compared with 9 of the 13 informative tumors with high instability ($P = 0.006$). Of our 13q microsatellite markers, this one is the closest to the *BRCA2* gene (19). Taken together, AI at either or both of the two *13q13* markers was significantly more frequent in the high instability group ($P = 0.037$). AI at either or both *13q13* and *RB* loci occurred significantly more often in the high instability group. When markers at both the *13q13* and *RB* loci were simultaneously informative, coordinate loss at both loci was observed in 10 of 16 cases. Most of these cases occurred in the high instability group (8 of 10). AI at the two 13q loci was often not contiguous. The *D13S218* marker, located between the *13q13* and *RB* loci, remained intact in 4 of the 7 cases which showed AI at *13q13* and *RB* loci and where markers at the three 13q sites (*13q13*, *RB*, and *D13S218*) were informative.

To better define the type of CIN measured by inter-SSR PCR, we performed flow cytometric analysis for aneuploidy in 19 of the tumors for which sufficient cellular material was present after DNA extraction. Six of 19 assessable tumors were aneuploid or tetraploid. Abnormal DNA ploidy was not correlated with high genomic instability (Table 2), whereas it was strongly correlated with AI at the *BRCA2* locus ($P < 0.005$).

DISCUSSION

The causes and mechanisms of genomic instability in breast cancer are at present not well defined. Indeed, the phenomenology of genomic instability in breast cancer is poorly studied. Sporadic breast cancers do not show MIN to any significant extent (3, 20). Our results confirm this finding because none of our 47 sporadic breast tumors showed MIN. The rarity of MIN breast cancers implies that other (non-MIN) forms of genomic instability underlie breast tumor progression. Indeed, previous karyotyping and allelotyping analyses confirmed that CIN is present in sporadic breast cancers (2, 3, 7). CIN may include many different forms of genomic alterations, some on a larger genomic scale (as detected by flow cytometry and karyotyping) and others on a smaller scale (smaller deletions, amplifications, and insertions).

We applied for the first time a genomic fingerprinting technique to sporadic breast cancers to quantify the degree of genomic instability present in these tumors. This study reveals the existence of genomic instability in almost all sporadic breast cancers, thus revealing the shortcomings of the popular division of sporadic breast cancers into two categories, diploid (genetically stable) and aneuploid (genetically unstable). Indeed, compared with sporadic colorectal cancers analyzed with the same technique (11), sporadic breast cancers show more alterations. Only one tumor did not show any alteration by this method. Furthermore, we found a bimodal distribution of instability in breast cancers; 27 (57%) of the cancers had few alterations, whereas 20 (43%) showed massive changes. Indeed, the differences between the two groups were almost immediately obvious on visual inspection. However, because of the representational nature of the technique, a few changes by inter-SSR PCR probably represent thousands of genomic alterations (12). Thus, many genomic alterations are present in the low inter-SSR PCR group of tumors as well. The bimodal distribution of instability may possibly reflect two different mechanisms of instability underlying breast tumorigenesis.

We have reported previously the lack of correlation between genomic instability as detected by inter-SSR PCR and fractional allelic loss in colorectal cancers (21). Similarly, we now report that aneuploidy as detected by flow cytometry is not correlated with inter-SSR PCR instability, suggesting that different forms of CIN are present in breast cancers. Flow cytometry is limited to the detection of large-scale chromosomal alterations, such as whole chromosomal deletions and duplications. To begin probing the nature of the changes detected by inter-SSR PCR, two changes detected by inter-SSR PCR were further analyzed. They were found to correspond to a locus of AI (a probable deletion) and locus of genomic amplification. Because inter-SSR PCR can detect amplifications and deletions in this study and in our previous work (12, 21), it is evident now that this technique detects changes which are mechanistically related to chromosomal breakage. Thus, inter-SSR PCR detects genomic alterations on a smaller scale than that which can be detected by flow cytometry. Clearly, gross chromosomal changes of the kind observed by flow cytometry are not the only chromosomal alterations present in sporadic breast cancers. It is tempting to speculate that, as in colorectal

cancer (22), each of these genomic instability phenotypes, low inter-SSR, high inter-SSR, and aneuploidy, may affect a different set of genes during breast tumor progression. Future investigations into breast tumorigenesis should perhaps consider these groups separately.

Recent studies have implicated BRCA1 and BRCA2, the recently discovered hereditary breast cancer genes, in the maintenance of genomic stability of cells (4–6). However, since their discovery, the perplexing finding has been the lack of somatic mutations in both genes in sporadic breast cancers. Nevertheless, AI has been reported in 18–60% of sporadic breast cancers at either of these loci (14–18). In our study, rates of AI were determined for loci surrounding both genes as well as for the *RB* locus, included because of its proximity to *BRCA2*. We found for the first time an association of AI at both *13q13* and *RB* loci with high genomic instability in sporadic breast cancers. Rates of AI at *13q13* and *RB* loci did not simply reflect higher levels of AI in the high instability group, *i.e.,* fractional allelic instability, because the rates of 17q AI, although greater in the high genomic instability group, were not significantly different between the two groups. Interestingly, AI at the *13q13* locus was strongly correlated with the presence of aneuploidy in the 19 tumors studied by flow cytometry. This confirms the results of a previous study (16) which showed such an association. Our results thus indicate that 13q involvement in genomic instability in sporadic breast cancers is associated with both gross chromosomal anomalies (aneuploidy) and intrachromosomal alterations (inter-SSR PCR). Because BRCA2 plays a critical role in the repair of double-strand DNA breaks, which are at the origin of intrachromosomal aberrations of the type detected by inter-SSR PCR, it may be that the *BRCA2* gene is the target of AI in this region. Although haplotype insufficiency at the *BRCA2* locus may have important ramifications for chromosomal stability (increased cellular radiosensitivity; Ref. 23), the relationship between AI and decreased gene expression at the *BRCA2* locus is far from proven (24). Confirmation of the role of the *BRCA2* gene will have to await a functional assay, which could be performed on breast tumors. A second possibility is that other genes in the *13q13* region (*e.g., FLT1*, *cyclin A1*, and *G-coupled protein receptor GREAT*) may be targeted by the 13q AI. A third possibility is that this genomic region may be particularly subject to both intrachromosomal and gross chromosomal alterations.

In conclusion, we have shown that genomic instability is present in virtually all sporadic breast cancers. Indeed, several (at least three) distinct phenotypes of CIN are present in these tumors. High and low intra-CIN seem to be operating independently of gross CIN. This finding is not surprising because the underlying mechanisms are probably different in nature; intra-CIN is likely attributable to mechanisms involving chromosomal breakage (*e.g.,* recombination anomalies and DNA repair dysfunction), whereas whole-CIN is likely attributable to dysfunction in the process of chromosomal segregation. Nevertheless, some overlap is probable as shown by the finding that two of these three groups are associated with alterations at the *13q13* locus. Further analysis of inter-SSR PCR alterations as well as the forthcoming availability of the complete genomic sequences of tumors and normal breast tissues will allow a further understanding of the nature of genomic instability in sporadic breast cancers, as well as of a possible role of BRCA2 in the triggering of genomic instability and subsequent breast tumor progression.

ACKNOWLEDGMENTS

We thank Drs. O-P. Kallioniemi, T. Bradley, S. Mousses, O. Salvucci, and M. T. Landi for their critical reading of this manuscript. We also thank Drs. A. Robidoux and J. Cantin for help in making the normal and tumor tissues available for the study.

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