Pergamon

European Journal of Cancer Vol. 31A, Nos 13/14, pp. 2330–2334, 1995

0959–8049/95 $9.50+0.00

0959-8049(95)00447-5

Original Paper

Infrequent Occurrence of Microsatellite Instability in Sporadic and Familial Breast Cancer

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Microsatellite instability was analysed in 93 primary breast tumours at 13 chromosomal loci frequently altered in breast cancer. RER (replication errors) were observed at a low (5%) frequency in sporadic, familial and hereditary breast tumours, as well as in breast tumours from patients with multiple primary cancers. Our study suggests that the RER+ phenotype is rare in breast tumours, and that breast cancer is not included in the hereditary non-polyposis colon cancer (HNPCC) syndrome. Moreover, the RER+ tumours revealed an atypical pattern of microsatellite alteration as compared with those usually seen in HNPCC tumours. In agreement with the findings in HNPCC tumours, all RER+ breast tumours were diploid, although having a similar frequency of allelic imbalance as RER− tumours. Thus, mismatch repair deficiency is rare in breast cancer, is most likely caused by somatic mutations, and possibly in a set of DNA repair genes different from that involved in the HNPCC syndrome.

Key words: microsatellite instability, breast cancer
Eur J Cancer, Vol. 31A, Nos 13/14, pp. 2330–2334, 1995

INTRODUCTION

THE DEVELOPMENT and progression of breast cancer is associated with a number of genetic alterations, including changes in oncogenes and tumour suppressor genes, for example, *ERBB2* amplification [1], or inactivation of *TP53* [2] in combination with mutations in several other specific genes, among others breast cancer susceptibility genes, *BRCA1* and *BRCA2* [3, 4]. Recently, a new, distinct type of genetic alteration, referred to as microsatellite instability, has been reported in several cancer types, particularly in colorectal cancer [5, 6]. What is observed is that novel bands of DNA, in the form of expansion or contraction, appear in one or both alleles of microsatellites in tumour DNA as compared with constitutional DNA. Microsatellite instability can occur during replication of repetitive sequences when two strands of DNA—the strand that is being copied and the new one being synthesised—slip relative to one another, resulting in small loops of unpaired DNA [7]. Normally, after replication, the mismatch repair system, which is carried out by products from four mismatch repair genes (MMR), i.e. *hMSH2*, *hMLH1*, *hPMS1* and *hPMS2* and probably a few other genes not yet known, would recognise mismatched base pairs, excise and replace them with correct nucleotides [8]. Obviously, the efficiency of heteroduplex repair is much reduced in cells with mutations in MMR genes. As a result, replication errors (RER) remain unrepaired and give rise to instability in many microsatellites, as typically seen in tumours from hereditary non-polyposis colon cancer (HNPCC) families and a proportion of sporadic colorectal cancer patients [9]. A recent study by Liu and associates [8] has shown that tumorigenesis associated with mutations in MMR genes usually results from inactivation of both alleles of the relevant gene. Regarding hereditary cases, e.g. in most HNPCC tumours, a germline mutation and an acquired somatic mutation account for loss of function in the relevant MMR gene, while in most sporadic RER+ tumours, cancer is likely to be associated with accumulation of two acquired somatic mutations in the MMR gene in a cell, or in other genes yet unknown [8]. Thus, microsatellite instability in patients with defects in the mismatch repair system is likely to be an indication that these individuals have a higher risk of accumulating mutations in other important genes, such as cancer-associated genes, which in turn can lead to predisposition to primary cancers at other organ sites, such as endometrium, stomach, pancreas, urinary tract, as has been seen in HNPCC families [10].

Moreover, the presence of a subclass of microsatellite instability, referred to as dynamic mutations, was shown to have diagnostic value in identifying a number of inherited neurodegenerative diseases, such as myotonic dystrophy, Kennedy's disease and fragile X syndrome [11]. This type of instability is characterised by extensive amplification of unstable trinucleotide repeats in germ line DNA from one generation to the next. For

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Received 20 Mar. 1995; accepted 12 Jun. 1995

example, in fragile X syndrome, the CCG trinucleotide is expanded from 30 to 2000 repeat units [12]. The mechanism and genetic basis of this alteration is relatively clear, but the involvement of MMR genes seems to be unlikely.

In several previous studies of HNPCC and sporadic colon cancer, a high frequency of microsatellite instability has been a consistent finding [6, 13], while the presence of microsatellite instability in other tumours such as lung and breast cancer has been controversial. In lung cancer, for instance, Peltomäki and associates detected microsatellite instability in 9% of 86 tumours [14], as compared to 34% of 38 cases reported by Shridhar and associates [15]. In all likelihood, the higher frequency seen in the latter study is due to the inclusion of multiple chromosome 3p microsatellites, suggesting that regions frequently involved in deletions are more often affected by microsatellite instability.

Similarly, in breast cancer, Wooster and associates reported microsatellite instability in 10% of 104 examined tumours [16], Lothe and colleagues [13] classified none of 84 tumours as RER+, and Han and colleagues [17] detected instability in 4% of 26 tumours. In contrast, Yee and associates detected instability in 20% of 20 examined tumours [18]. In view of these varying results, we decided to investigate the frequency of the RER+ phenotype in breast cancer, by studying 93 tumours using 13 microsatellite markers from chromosomal loci reported to be frequently deleted (loss of heterozygosity) in breast cancer. In order to assess whether incidence of microsatellite instability was correlated to inheritance in breast cancer, we subdivided patients in sporadic, familial and hereditary subgroups. Moreover, we studied a small group of cancer-prone patients with multiple primary cancers, including endometrial, ovarian, cervical or gastrointestinal tract cancer, in addition to breast cancer, to see whether the RER+ pattern and frequency vary between these two groups.

PATIENTS AND METHODS

Patients

First group. The analysis is based on 70 women with a single sporadic, familial or hereditary primary invasive breast cancer. All patients were from the Southern Swedish Health Care Region, diagnosed and surgically treated for primary invasive breast cancer between 1983 and 1993 (Table 1). None of the patients was treated with radiation or chemotherapy before resection. In order to define different subgroups within this group, the following criteria were used:

Subgroup 1: Breast cancer in patients without any kind of cancer in the family were classified as sporadic unilateral breast cancer, $n = 30$.

Subgroup 2: Breast cancer in patients with only one first degree relative with breast cancer was classified as familial, $n = 20$.

Subgroup 3: Breast cancer in patients who had two or more first degree relatives with breast and/or ovarian cancer, at least one of them with early onset of (before age 50) breast cancer, was classified as inherited, $n = 20$.

Second group. Breast tumour samples from 23 female patients with multiple primary cancer were analysed. Patients in this group had been treated by surgery, irradiation or chemotherapy for 1–3 primary invasive cancers, developed in the endometrium, ovary, cervix and/or gastrointestinal tract during 1960–1994, in addition to primary invasive breast cancer developed between 1989 and 1994 (Table 1). Among this group, 7 patients had breast cancer as their first malignancy, whereas the remainder had been treated for other cancer types before developing breast cancer. Information about multiple cancers in this group was obtained from the Regional Tumour Registry in Lund.

Table 1. Characteristics of 70 patients with a single breast cancer (SBC) and 23 patients with breast cancer and primary cancers in other organ sites (endometrium, ovary, cervix and gastrointestinal tract (MC). Only the breast tumours were available for investigation

	SBC			MC*
	Sporadic ($n = 30$)	Inherited ($n = 20$)	Familial ($n = 20$)	Sporadic ($n = 23$)
Age				
≤ 40 years	0	10	2	2
> 40	30	10	18	21
Clinical stage				
I	13	9	7	13
II	17	9	10	8
III	—	2	2	2
IV	—	—	1	—
Lymph node status				
Positive	16	12	13	12
Negative	14	8	7	11
ER status				
Positive	28	12	14	16
Negative	2	8	6	7
PgR status				
Positive	22	12	14	14
Negative	8	8	6	9
Tumour size				
≤ 20 mm	6	10	9	14
> 20 <50 mm	13	6	8	7
≥ 50 mm	1	4	3	2
Histopathological type				
Ductal	24	12	17	17
Lobular	1	2	2	4
Other†	5	6	1	2

*A total of 49 tumours were observed in the 23 female patients, 23 in breast, three in the endometrium, three in the ovary, 10 in the cervix and 10 in the gastrointestinal tract. †Tubular, medullar, mucinous, mixed types. ER, oestrogen receptor; PgR, progesterone receptor.

Tumour tissues and methods

A total of 93 breast cancer biopsies and paired blood samples were obtained from patients who had been surgically treated for primary invasive breast cancer in the Southern Swedish Health Care Region. The tumour samples were frozen in liquid nitrogen immediately after surgery and stored at −80°C until use. All frozen tumour specimens were ground in a Braun Microdismembrator (Melsugen, Germany) and the resulting powder was suspended in lysis buffer [0.5% sodium dodecyl sulphate (SDS), 0.3 M sodium acetate, 5 mM EDTA, pH 8.5] and centrifuged for 30 min. The supernatant cytosol was analysed for oestrogen (ER) and progesterone receptor (PgR) content by enzyme immunoassay with a cut-off value of 25 fmol/mg protein as described before [1], while the nuclear pellet was used to extract DNA according to the conventional Proteinase K and phenol/chloroform method. The DNA content and ploidy status of samples that showed microsatellite instability were analysed by flow cytometry (FMC), after staining of nuclei with propidiumiodine as described previously [19]. Tumour DNA content was defined as diploid if there was only one stem population, otherwise as non-diploid [20].

Microsatellite analysis

DNA from blood (peripheral white cells) and paired tumours were analysed for microsatellite instability at 13 different microsatellite repeat polymorphisms (Table 2) by PCR amplification. The selected microsatellites were di-, tri- and tetranucleotide repeats from chromosomal loci that have been reported to be frequently deleted (LOH) in breast cancer. All used markers were obtained as MAPPAIRS (Research Genetics, Huntville, Alabama, U.S.A.) except *vWFa*, *AR* and *DM* markers [16]. The forward primers were 5′ end-labelled with (γ^{32}P)-ATP using T4-polynucleotide kinase (Promega). The following PCR protocol was used: 80 ng of genomic DNA template, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 0.75–1.5 mM $MgCl_2$, 250 μM of each dNTP, 0.8 μM of reverse primer, 0.4 μM of unlabelled plus 0.4 μM labelled forward primer and 0.6 units of Taq polymerase (Boehringer Mannheim) in a 25 μl reaction. PCR conditions consisted of a denaturation step, 5 min in 94°C and 30 cycles at 95°C for 40 s, 60°C for 30 s, 72°C for 30 s. The PCR was completed by 10 min in 72°C.

Amplified PCR products were diluted 6-fold in loading buffer [95% (v/v) deionised formamide, 10 mM NaOH, 0.1% (w/v) bromophenol blue, and 0.1% (w/v) xylene cyanol], denatured at 93°C for 3 min and electrophoresed on a 6% polyacrylamide gel containing 7 M urea, to allow proper separation of polymorphic alleles. The gels were exposed to a X-ray film for 6–24 h at −70°C. The size of the separated alleles was then compared between tumour tissue and paired normal DNA.

Seqeuencing of microsatellites

In order to confirm the suspected microsatellite alterations, the exact sequence change was determined by cutting out and sequencing two typically shifted bands, one expansion and one contraction detected in *vWFa* and *AR* loci, respectively, in addition to two parental bands equivalent to those appearing in normal DNA. The desired fragments were amplified by using 5′ end-biotinylated forward primer. A single stranded template for sequencing was produced by binding the amplified biotinylated PCR products to avidin-Dynabeads (Dynal). The sequencing reaction was performed by the dideoxy chain termination method described by Sanger and associates [21].

Table 2. Summary of used markers and alterations obtained in microsatellite repeat loci for both patients with single breast cancer (SBC) and multiple cancers in addition to breast cancer (MBC)

Polymorphic short tandem repeat loci studied					
Locus	Repeat sequence	Chromosomal loci	Allele size (bp)	Instability SBC	MBC
D2S132	GT	2p21	197–227	—	—
ESR	TA	6q24–27	162–194	—	—
THRB	CA*	3p24.1	197–209	—	—
D3S1067	AC	3P14.3–21.1	95	—	—
D16S303	GT	16q24.3	113	1	—
AR	CAG	Xq11–12	195–225	1	—
DM	CTG	19q13.3	128–191	1	—
D6S366	AAT	6q21-qter	138–162	—	—
OF2	AAAG	17q12–21	260	—	—
vWFa†	TCTA	12p12-pter	154–174	1	—
vWFb	TCTA	12p12-pter	138–162	1	—
N-MYC	TATG	2p24	119–131	—	—
MYCL1	AAAG	1p32	140–209	2	1

*(CA)3CGTG(CA)7T(AC). † vWFa and vWFb are located within an intron of the gene encoding Willebrand's factor and are separated by 700 bp.

RESULTS

In the first group of patients, a novel band of DNA not present in the paired normal DNA, was detected in four (6%) of 70 tumours studied. Of 13 analysed microsatellite loci, alterations were observed in seven different loci, detected with di-, tri- and tetranucleotide repeat markers, all confirmed by replicate examinations (Figure 1). Three of the four samples showed an instability in a single locus in the form of expansion, whereas the fourth tumour exhibited expansion as well as contraction with four different markers. Of 13 used polymorphic markers, five were dinucleotide repeats which detected a single RER+ tumour (one of 350 i.e. 70 samples and five different markers), whereas more cases of instability were observed with tri- (two of 210) and particularly by tetranucleotide repeat (four of 350) markers (Table 2).

The detected RER+ phenotype was equally distributed among the three subgroups: in sporadic 6.6% (two of 30), in hereditary 5% (one of 20) and in familial 5% (one of 20).

Frequency of microsatellite instability in patients of the second group was similar to that of the first group, i.e. we detected microsatellite instability in one of the 23 (4%) examined tumours, in form of an expansion at the *MYCL1* locus (Table 2). This RER+ tumour, a sporadic case, was from a patient who had been treated for ovarian and ileum cancer before developing breast cancer, and who is still alive and well after 72 months.

Generally, a higher frequency of allelic imbalance (allele gain or loss) was observed for tumours from patients with multiple cancers, i.e. 26 sites were affected in 23 tumours as compared with 43 sites in 70 tumours in the first group. Moreover, allelic imbalance occurred at a similar frequency in both RER+ and RER− tumours in both groups, particularly manifest for tri- and tetranucleotide loci. Some sites were more frequently affected than others, for example, the androgen receptor gene, *AR* (Xq11), which showed allelic imbalance at 22% of 69 altered sites.

All five RER+ tumours were clinical stage II and DNA diploid that indicates a notable association between RER+ phenotype, clinical stage and DNA ploidy status in breast cancer. Finally, three of the five RER+ tumours were positive for ER and PgR.

Sequence analysis of abnormal repeats

To investigate the nature of expansion or contraction observed in RER+ tumours in breast cancer and to verify that these sequence changes did not arise by, for example, mitotic recombination or other mechanisms which could give rise to genetic alterations [22, 23], we sequenced two RER+ samples, one contraction and one expansion. The origin of the additional band was determined by comparing the relative allele intensity between tumour and normal DNA. The allele with reduced band intensity is the allele from which the extra band is assumed to originate [16]. The nucleotide sequence of tumour 1 with a contracted band detected by the *AR* marker (Figure 1) had two bands identical in size with corresponding normal DNA (21 and 22 CAG units) and also a smaller band with 11 repeat units (Figure 2). In tumour 3, the upper parental allele (less intensive band with 13 TCTA) has been expanded by one repeating unit. Both parental alleles (11 and 13 units) were identical to those which appeared in normal DNA (sequence is not shown). Thus,

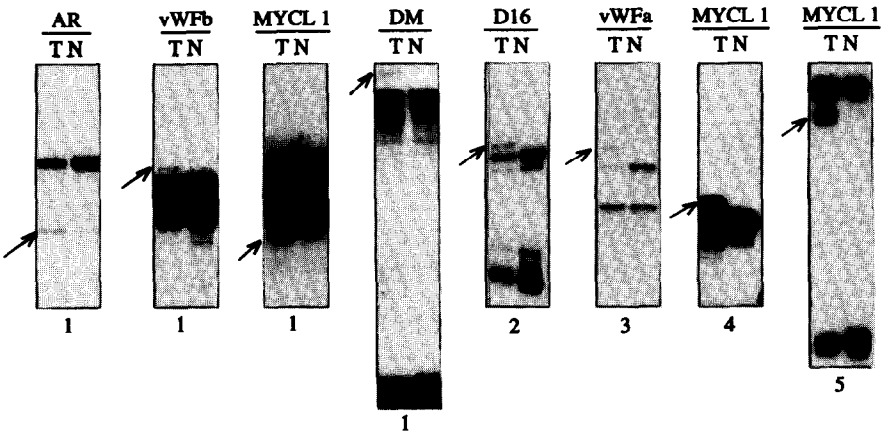

Figure 1. The microsatellite repeat patterns at investigated loci are shown for tumours 1–5. Tumour 5 is from a multiple cancer patient. N, normal (blood); T, tumour DNA. The additional bands present in tumours, but not in normal DNA, are shown with arrows.

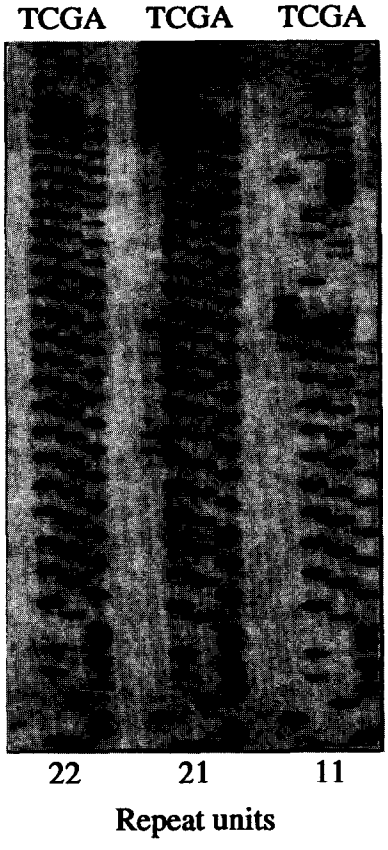

Figure 2. Nucleotide sequence of the androgen receptor (CAG)n repeat locus for tumour 5, confirming an alteration in the number of CAG repeat units in the contracted band in comparison with those present in two parental bands.

the changes in these tumours seem to be an alteration in the number of repeat units, i.e. as expansion or contraction.

DISCUSSION

Our data indicate a low incidence of microsatellite instability in breast cancer. Only 5% of 93 analysed tumours had evidence of being RER+, in spite of using markers for chromosomal regions commonly involved in loss of heterozygosity in breast cancer. We found no marked differences between patients with sporadic, familial and hereditary breast cancer, nor did patients with multiple primary cancers differ in this respect. The similar low frequency for both sporadic and familial cancers indicates that germ line mutations in the MMR genes are rare in hereditary breast cancer and support the notion that breast cancer is not involved in the HNPCC syndrome [13]. The tumorigenesis associated with the mismatch repair system in breast cancer can be the result of inactivation of both alleles in the relevant MMR gene by, for example, two acquired somatic mutations. The fact that tumour 1 (Figure 1) showed microsatellite instability in four different loci may reflect a typical complete inactivation of mismatch repair in this breast tumour, whereas tumours with alterations in a single locus may have only a reduced mismatch repair capacity. If heterozygous for the relevant gene of the mismatch repair system in breast cancer then this could contribute to the reduction of repair capacity.

Alternatively, the microsatellite instability in breast cancer has a pattern different from that observed in RER+ tumours, such as those from HNPCC families. While this is seen for multiple dinucleotide loci in, for instance, colon cancer, the microsatellite instability in breast cancer usually affects one single locus and mainly the tri- or tetranucleotide repeats. Moreover, in breast cancer, it always appears as a single new band, as compared with the ladder pattern of new alleles typically seen in HNPCC tumours [10]. Finally, the inverse correlation between loss of heterozygosity (LOH) and RER+ phenotype seen in colon cancer has not been observed in breast cancer [13]. Accordingly, together these differences indicate that the basic genetic mechanism for microsatellite instability seen in breast cancer is distinct from that observed in tumours from HNPCC families. Moreover, the fact that three of the eight detected extra bands were in the *MYCL1* locus (37%) and the frequent occurrence of LOH in the androgen receptor (AR) locus indicate that some alterations are tumour-type specific.

In a previous study, Umar and associates [24] showed that extract from an endometrial cell line known to be mutated in one of the *hMSH2* alleles could efficiently repair a heteroduplex containing loops consisting of five bases. This finding showed that distinct classes of repair system operate depending on the loop size and the surrounding sequence. Thus, instability in tri- and tetranucleotide repeats seem to be associated with mutation in unknown genes, operating in a repair system other than that reported for colon cancer. For instance, the extra band seen in

tumour 1 of the present study, would have arisen from an unrepaired loop of approximately 30 nucleotides. It has recently been assumed that defects in the replication system, such as mutations affecting exonuclease activity of DNA polymerase, can lead to reduced replication fidelity and allow alteration in several microsatellite loci [25]. Furthermore, a study by Glebov and associates has shown a significant correlation between *TP53* mutation and altered allele size (microsatellite instability) in familial and sporadic breast cancer [26]. This observation, in combination with the finding of the present study that both microsatellite alterations and allelic imbalance at multiple sites are detected in a single diploid tumour, suggests that *TP53* mutations may occur early in breast cancer development, predisposing the cell to further genetic changes, which manifest as allelic imbalance and microsatellite instability.

The present study shows that microsatellite instability is an infrequent event in breast cancer. The RER+ phenotype had no relationship with the family history of carcinomas in this study. Tri- and tetranucleotide instability were more frequent than dinucleotide repeats, and often appeared for one single locus. Our result also indicates that a correlation may exist between RER+ and DNA diploid status, while no association was found as regards hormone receptor status. The observation that the RER+ is associated with good prognosis in colorectal cancer by Lothe and associates [13] may also be true for breast cancer, since all patients in this study with RER+ phenotype are still alive and well, although mastectomy was carried out between 1983 and 1989.

A similar RER+ incidence was also observed in tumours from multiple cancer patients, which indicates that the mismatch repair system in breast cancer was affected neither by various treatments nor by cancer in other organs in these patients.

Further investigations could give some indication for the low frequency of RER+ in breast cancer, for example, screening for mutations in *TP53* in RER+ samples could clarify the pathway in which the tumour cells accumulate mutations and become unstable.

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Acknowledgements—This work has been supported by grants from the Swedish Cancer Society, the Nordic Cancer Union, the Mrs Berta Kamprad Cancer Foundation, the Hospital of Lund Foundation and the Medical Faculty, University of Lund.