



Report

Microsatellite instability markers in breast cancer: A review and study showing MSI was not detected at 'BAT 25' and 'BAT 26' microsatellite markers in early-onset breast cancer

Shoo Peng Siah^{1,2}, Diana M Quinn¹, Graeme D Bennett², Graeme Casey², Robert LP Flower¹, Graeme Suthers³, and Zbigniew Rudzki²

¹School of Pharmacy and Medical Science, University of South Australia, City East Campus, Adelaide; ²Molecular Pathology, Institute of Medical and Veterinary Science, Adelaide; ³South Australia Clinical Genetics Services, Women's and Children's Hospital, North Adelaide, Australia

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Summary

Microsatellite markers may provide evidence of faulty DNA mismatch repair (MMR) via the detection of microsatellite instability (MSI). The choice of microsatellite markers may impact on the MSI detection rate. In hereditary non-polyposis colon cancer (HNPCC), several informative microsatellite markers have been recommended. Two of these, BAT 25 and BAT 26, are quasi-homozygous, enabling analysis of tumour DNA in the absence of paired normal DNA. Sixty-six breast cancer patients under 45 years of age at diagnosis were examined for MSI at BAT 25 and BAT 26. Tumour DNA was extracted from paraffin-embedded tissue. No MSI was detected at the BAT 25 or BAT 26 loci. An additional five microsatellite markers, known to be informative for HNPCC, were examined for MSI in these patients. Apparently-normal profiles were achieved. A tabulated survey of 306 microsatellite markers used to detect MSI in breast cancer revealed that only 35.5% of markers detected MSI at an average rate of 2.9%. The MSI detection rate at the specific HNPCC markers varied from 0% to 10% in breast cancer, with D175250 and TP53 being the HNPCC markers most suitable for analysis of breast cancer. The size of the microsatellite marker's repeat unit did not impact on MSI detection rates. Compiled data from large studies ($n > 100$) revealed D115988 as the marker with the highest MSI detection rate. Genomic instability pathways of carcinogenesis, characterised by MMR defects and MSI, appear to play a role in the genesis of some breast cancer types.

Introduction

Microsatellites are repeat regions of one to six nucleotide units that occur primarily in non-coding regions of DNA [1]. The number of microsatellite repeat units located at a given locus is genetically determined [2]. These highly repetitive regions of DNA are difficult for DNA polymerase to faithfully reproduce during DNA synthesis [1]. Slippage may occur with copies of the repeat unit being inserted or deleted, thereby altering the size of the locus [1]. Normally, the integrity of the genome is monitored by a number of mechanisms,

one of which is involved in the detection and correction of DNA mismatches – the DNA mismatch repair (MMR) mechanism.

Defects in DNA MMR mechanisms have been shown to be a feature of the familial disorder hereditary non-polyposis colorectal cancer (HNPCC) [1, 3]. A common characteristic of this defect is the observation of instability at microsatellite markers in tumour DNA. Microsatellite instability (MSI) can be detected by demonstration of variability in the number of repeat units in selected microsatellite markers following amplification using polymerase chain reaction (PCR)

between tumour and normal DNA [3]. MSI at multiple microsatellite markers has been identified in up to 90% of the patients diagnosed with HNPCC [3]. It has also been reported in some breast cancer patients with variable frequency (0–33%) [4, 5]. Although a proportion of this variability may be due to non-uniform sample selection and differing analysis criteria, it is apparent that the choice of microsatellite markers to be analysed plays a significant role in the rate of detection of MSI. A review of microsatellite markers that have been used to screen for MSI in breast cancer has been compiled in conjunction with this study and posted on the internet [6].

In 1996, Risinger et al. proposed that breast cancer was an integral tumour in patients with HNPCC [7]. This study involved screening of 21 microsatellite markers for MSI in five HNPCC patients with breast cancer and subsequent sequencing of some MMR genes. MSI was detected in all patients at various microsatellite markers and molecular defects in MMR genes were established in two patients. It was proposed that the type of MMR defects detected in HNPCC may also be a cause of hereditary breast cancer [7]. The aim of this study was to investigate whether early-onset breast cancer had a similar process of tumourigenesis as detected in HNPCC, by performing MSI analysis using markers effective for the detection of MSI in colorectal cancer [8, 9, 10, personal observations].

BAT 25 and BAT 26 are mono-nucleotide microsatellite markers located on chromosome 4q12 and 2p15, respectively. The wild-type BAT 25 and BAT 26 are expected to be homozygous and monomorphic in most individuals and hence will give a single peak following PCR amplification. The sizes of the products following PCR amplification of these microsatellite markers are generally stable in the normal DNA of individuals (variation of < 2 bp) [9, 11, 12] but variable in tumour DNA from HNPCC patients with defective MMR function (variation of 4–16 bp) [9–11]. BAT 25 and BAT 26 were selected for our investigation of MSI in early-onset breast cancer tumour DNA, without corresponding normal patient DNA, as the detection of heterozygous alleles (two or more peaks) would be indicative of MSI [9].

Five additional microsatellite markers, namely BAT 40, D2S123, D10S197, D17S579 and D18S34, were also investigated to increase the chance of detecting MSI. As these microsatellite markers may be either homozygous (giving one peak following amplification) or heterozygous (giving two peaks), the detection

of three or more peaks was the criterion for inference of MSI for these markers.

Methods

Samples

Tissues from 66 South Australian women diagnosed with breast cancer at or under the age of 45 years were selected from the South Australian Cancer Registry. A specialist tissue pathologist identified appropriate tumour sections and two 10 µm paraffin-embedded tumour sections were prepared.

Blood and colorectal tumour tissue DNA, from a HNPCC patient with a truncating *hMLH1* germline mutation, were obtained from the Molecular Pathology Laboratory at the IMVS. This was used to confirm the sensitivity of the experimental system.

DNA extraction

Paraffin was removed from tissue section using three xylene washes, one absolute ethanol wash, and one wash, with sterile MilliQ water (1 ml solvent per wash, 10 min incubation at room temperature). Tissue samples were digested with Proteinase K (Merck) for two days at 55°C and DNA extracted using a salt extraction method [13].

Microsatellite marker analysis

Primers published for seven microsatellite markers (BAT 25, BAT 26, BAT 40 [14] D2S123 [15], D10S197 [15], D17S579 [16], D18S34 [17]), were 5'-end labelled during synthesis with HEX amidite or 6-FAM amidite dyes (Applied Biosystems, Foster City, CA). DNA (2.5–10 ng) was amplified in a standard 50 µl reaction mix (200 µM of each dNTP, 2 mM of MgCl₂, 5 µM of forward fluorescent and reverse non-labelled primers, 0.5 U of AmpliTaq[®] Gold DNA polymerase with 1× reaction buffer [Perkin Elmer/Cetus]). Cycling conditions included an initial denaturation at 95°C for 12 min followed by 40 cycles of 30 s at 95°C, 30 s at 55°C, and 90 s at 68°C, with a final extension of 10 min at 68°C.

PCR products were electrophoresed on a 6% polyacrylamide gel, detected using an ABI Prism[™] 377 DNA Sequencer (Perkin Elmer, Foster City, CA) and analysed by the ABI Prism GeneScan[™] and Genotyper[®] Analysis software (Applied Biosystems,

Foster City, CA). Patients were scored as homozygous when a single peak was observed in the electropherogram, or heterozygous when two distinct peaks were detected. Microsatellite analysis of each of the samples was performed on at least three occasions.

Scoring MSI

The detection of MSI at two or more microsatellite markers was required to classify a tumour as exhibiting MSI. For the normally homozygous BAT 25 and BAT 26 microsatellite markers, the detection of two or more peaks following amplification of tumour DNA was regarded as indicative of MSI at this marker. For the other five microsatellite markers, the detection of three or more peaks following amplification of the DNA was the criterion for identification of MSI at these markers.

Results

MSI control (HNPCC patient)

The sensitivity of the assay for detection of MSI was demonstrated by detection of allelic changes in a HNPCC patient with a germline *hMLH1* mutation. For all seven microsatellite markers, GeneScanTM and Genotyper[®] analysis of PCR amplified products from the HNPCC tumour DNA revealed at least one more peak than observed in the corresponding non-tumour DNA. This was consistent with the assay being sufficiently sensitive for the detection of MSI. As MSI was detected at greater than two microsatellite markers, this enabled the classification of the HNPCC tumour as MSI positive.

Microsatellite marker analysis in breast cancer

Breast tumour samples from 66 women under 45 years of age at first diagnosis were examined for MSI at seven microsatellite markers. At the BAT 25 and BAT 26 microsatellite markers, in which normal individuals are usually homozygous, a single peak was found in all 66 of the tumour DNA samples examined. For the other five microsatellite markers, in which normal individuals may be homozygous or heterozygous, either one peak (58/66, 87.9%) or two peaks (8/66; 12.1%) were detected. There was no detection of three or more alleles for any of the five microsatellite markers examined. As MSI was not detected at greater than

two microsatellite markers in any of the patients, no tumours were classified as MSI positive.

Discussion

Microsatellite marker analysis has been used extensively to detect evidence of defective repair of DNA synthesis errors. The basis for most of these analyses was the discovery of extensive MSI and mutated MMR genes associated with the hereditary colon cancer syndrome, HNPCC [1, 3, 18–20]. In these kindreds, ineffective detection and repair of DNA mismatches results from a failure of the repair process [19, 20]. As DNA synthesis of repeat sequences within microsatellite regions is error-prone [21], without repair, DNA defects accumulate. Accumulation of these errors in crucial genes, such as growth regulatory genes, contributes to tumour initiation and progression [1, 14].

To determine if mismatch repair defects were contributing to a proportion of early-onset breast cancer cases, we examined breast cancer tissue from women under the age of 45 for the presence of MSI. The microsatellite markers chosen had been successfully used in our laboratory to detect MSI in HNPCC and have been shown to be as effective in detecting MSI in HNPCC as those recently recommended by the National Cancer Institute Workshop on microsatellite instability [8] (data not shown). No evidence of MSI was detected at any of the seven loci examined in our 66 breast cancer patients. Other studies examining the microsatellite markers that we used, revealed that only 42 of 743 analyses (5.7%) had MSI in breast cancer [5, 7, 10, 22–33]. This was substantially lower than the reported MSI detection rate in sporadic colorectal cancer (17%) [34]. From this it appeared that MSI was not a common feature of breast cancer.

These observations prompted us to investigate the usefulness of MSI analysis in breast cancer by reviewing the current literature. A survey of 43 publications and 18,055 microsatellite analyses from breast tumours revealed that greater than 300 different microsatellite markers have been used to detect MSI [6]. This illustrates the popularity of this type of investigation. While the overall positive detection rate was 2.9%, compared to 17% in sporadic colorectal cancer [34], approximately 65% of the microsatellite markers we reviewed for breast cancer showed no MSI [6]. This suggested that the screening of microsatellite

Table 1. Compiled rates of MSI detection in breast cancer at defined HNPCC microsatellite markers

HNPCC marker ^a	Suggested by ^b	Compiled rate of MSI in breast cancer (%) ^c	References
D17S250	[8]	10.0% (n = 180)	[5, 30–32, 36]
TP53	[37]	7.5% (n = 200)	[25, 29, 31, 32, 50, 51]
D2S123	[8, 37]	5.2% (n = 288)	[5, 7, 23–26], This study ^e
BAT 25	[8]	3.2% (n = 95)	[7, 22], This study
BAT 26	[8, 37]	2.2% (n = 179)	[7, 10], This study
D5S346	[8]	0.0% (n = 22)	[52]
FGA	[37]	0.0% (n = 24)	[53]
D18S35	[37]	NR ^d	

^a“HNPCC marker” refers to the microsatellite markers that have been suggested for the detection of MSI in patients with hereditary non-polyposis colorectal cancer (HNPCC).

^b“Suggested” by indicates the article(s) that propose the use of specific microsatellite markers to detect MSI in HNPCC.

^c“Compiled rate of MSI in breast cancer” refers to the percentage of MSI positive patient samples, as detected by researchers at a given microsatellite marker, out of the combined number of patients surveyed. Full details available at URL: <http://Drmserv1/microsatellite markers/> [6].

^d“NR” indicates that none of the articles reviewed on breast cancer microsatellite studies included this microsatellite marker.

^e“This study” indicates that the calculated rate (%) of MSI in breast cancer includes the data for the 66 patients investigated in this study.

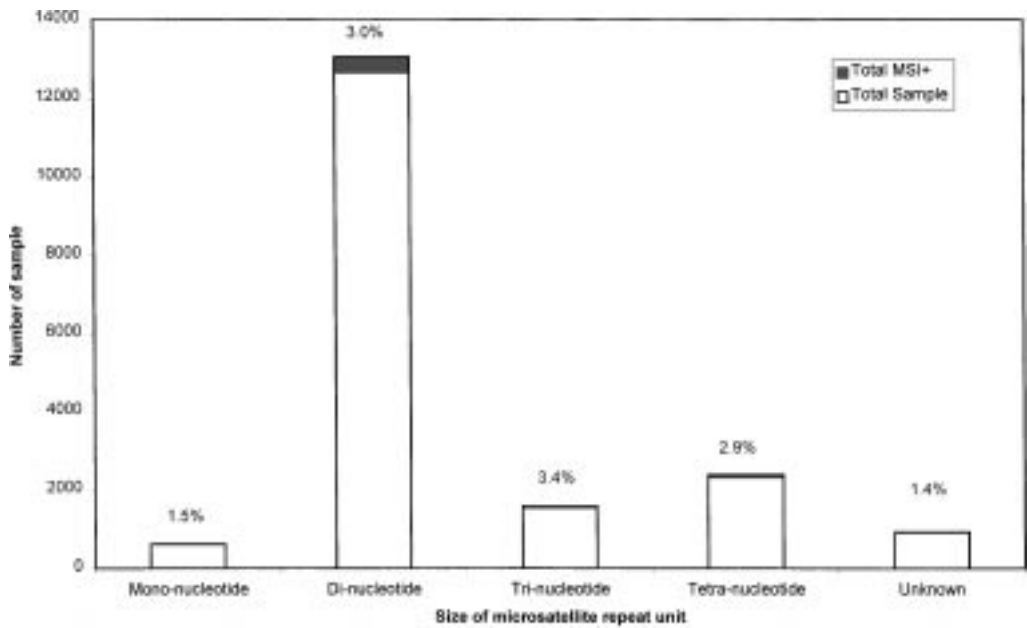


Figure 1. MSI analysis of breast cancer by size of microsatellite marker repeat unit. Data was collected from a survey of 306 microsatellite markers and 18,055 DNA samples [6] and organised by size of the microsatellite marker repeat unit used to detect MSI in breast tumour samples. The unknown column represents undefined microsatellite markers, in which the repeat unit was not specified. The number of DNA samples that had detectable MSI are shaded (top, percentage shown above) and the number of samples in which MSI was not detected are unshaded (bottom).

markers in breast cancer had largely been uninformative. No allowance for different tumour types has been made in this survey; however, evidence that different types of tumour have different MSI rates has been

Table 2. Compiled rates of MSI detection at defined microsatellite markers in breast cancer ($n > 100$)

Marker ^a	Total % ^b	Reference
D11S988	17.7 ($n = 124$)	54, 55 ^c
ACTC	14.0 ($n = 107$)	5, 33, 36
D21S1436	13.9 ($n = 115$)	31, 52, 55 ^c
D8S135	10.9 ($n = 110$)	23, 28 ^c
D17S250	10.0 ($n = 180$)	5, 30–32, 36
D2S443	9.7 ($n = 113$)	31, 52, 55 ^c
D17S518	9.0 ($n = 100$)	28 ^c
TP53	7.5 ($n = 200$)	25, 26, 29, 31, 32, 50
D1S104	6.5 ($n = 107$)	5, 26, 36, 42
D17S807	6.4 ($n = 126$)	28, 33 ^c
DM-1	5.8 ($n = 359$)	7, 24, 40, 22, 35, 55 ^c
D17S579	5.7 ($n = 245$)	29–32, 51, This study ^d
D2S123	5.2 ($n = 288$)	5, 7, 23–25, 51, This study
D10S197	5.0 ($n = 259$)	7, 18, 23, 27, 28 ^c , This study
D3S1611	4.9 ($n = 103$)	5, 56 ^c
D18S34	4.9 ($n = 267$)	5, 33, 36, 28 ^c , This study
D6S193	4.0 ($n = 126$)	22, 35, 57 ^c
Nm23-H1	3.8 ($n = 185$)	23, 58
AR	3.6 ($n = 309$)	24, 33, 40, 35, 55 ^c
D16S301	3.3 ($n = 184$)	24, 29
MYCL1	2.9 ($n = 139$)	33, 40, 42
D3S1514	2.5 ($n = 163$)	59, 60
D1S213	2.4 ($n = 124$)	42, 28 ^c
BAT 26	2.2 ($n = 179$)	7, 10, This study
D17S855	2.2 ($n = 316$)	29–31, 51, 59, 60
HUMARA	1.8 ($n = 109$)	59
APOC3	1.7 ($n = 179$)	61 ^c
D16S413	1.6 ($n = 192$)	24, 62, 22, 35 ^c ;
VWFFa	1.5 ($n = 197$)	24, 40
ESR	1.4 ($n = 220$)	40, 22, 35, 57 ^c
THO1	1.4 ($n = 146$)	59, 60
FABP	1.3 ($n = 157$)	59, 60
D9S254	1.2 ($n = 169$)	59, 60
D11S1778	1.1 ($n = 179$)	61 ^c
DXS981	1.0 ($n = 104$)	24
VWFFb	1.0 ($n = 197$)	24, 40
D2S136	0.9 ($n = 109$)	25, 29, 51
D11S1818	0.6 ($n = 179$)	61 ^c
D17S559	0.6 ($n = 166$)	59, 60
D11S1294	0.6 ($n = 179$)	61 ^c
D16S303	0.4 ($n = 277$)	24, 29, 40

Table 2. (continued)

RII (1)	0.0 ($n = 111$)	63, 28 ^c ;
D1S216	0.0 ($n = 107$)	18, 27, 42
D3S1067	0.0 ($n = 199$)	25, 29, 40
c-myc	0.0 ($n = 104$)	24
D11S29	0.0 ($n = 108$)	64, 65
D11S927	0.0 ($n = 179$)	61 ^c
D11S1325	0.0 ($n = 179$)	61 ^c
D11S1347	0.0 ($n = 179$)	61 ^c
D11S1816	0.0 ($n = 179$)	61 ^c
D11S1819	0.0 ($n = 179$)	61 ^c
D11S2179	0.0 ($n = 179$)	61 ^c
D16S588	0.0 ($n = 104$)	24
GH1	0.0 ($n = 103$)	58, 66

^a“Marker” is the microsatellite marker name commonly used to in the literature.

^b“Total %” refers to the percentage of the combined MSI positive samples from the combined number of amplifiable DNA samples analysed for a given marker in this survey.

^cPersonal communications with authors were undertaken to confirm MSI rates.

^d“This study” indicates that the compiled MSI rate (%) includes the data for the 66 patients investigated in this study.

reported [5, 22, 35, 36]. This survey does not include studies examining loss of heterozygosity or MSI in breast cancer precursor lesions. A further constraint was that, in some cases, we were unable to extract desired information from the published material. In the majority of these cases the data presented is the result of personal communications.

A choice of informative microsatellite markers for the detection of MSI in tumour tissue is imperative. Reviews and workshops for the evaluation of HNPCC have suggested the use of selected microsatellite markers for routine screening of MSI [8, 37, 38]. HNPCC is the clearest example of the process by which DNA mismatch repair defects result in carcinogenesis, a process that is characterised by MSI [1]. A review of microsatellite markers that have been used to detect MSI in breast cancer [6] revealed the rate of MSI detected at defined HNPCC microsatellite markers [37, 38] varied from 0.0% (D5S346 and FGA; $n = 46$) to 10.0% (D17250, $n = 180$) (Table 1). One recently suggested HNPCC marker, D18S35 [37], does not appear to have been studied as a marker of MSI in breast cancer. As the overall detection rate of MSI in breast cancer is low (2.9%), it is reasonable to conclude that only two of the suggested HNPCC markers, D17S250 (10.0%) and TP53 (7.5%), are useful for the detection of MSI in breast cancer.

It has been previously reported that the rate of spontaneous mutation in microsatellites with tri-nucleotide and tetra-nucleotide repeat units is up to 50 times greater than microsatellites with di-nucleotide repeat units [15, 39]. This implies that microsatellite markers that have tri- and tetra-nucleotide repeat units may be more effective for detecting MSI [40]. Our survey of microsatellite markers in breast cancer [6] did not substantiate this as the MSI detection rates at mono-nucleotide, di-nucleotide, tri-nucleotide and tetra-nucleotide repeat units were similar (Figure 1). Nevertheless, this is at variance with that observed in HNPCC, for which mono-nucleotide microsatellite markers offer the best MSI detection rates [8].

Sample size influences the reliability of experimental data. By restricting this survey of microsatellite markers used in breast cancer to those with greater than 100 DNA samples analysed, a more accurate picture of potentially useful markers can be drawn (Table 2). The microsatellite marker that was most informative was D11S988, with an MSI positive detection rate of 17.7% ($n = 124$), comparable to the MSI detection rate found in sporadic colorectal cancer [34]. This marker is located near a tumour-suppressor gene region at 11p15 that has been shown to exhibit defects in several cancer types, including breast cancer [41]. The next most efficient microsatellite markers for detecting MSI in breast cancer were ACTC (14.0%, $n = 107$), which is a CA repeat located within intron 4 in the cardiac muscle actin gene [33]), followed by D21S1436 (13.9%, $n = 115$), a GGAA repeat located on chromosome 21. Although the MSI detection rates of these three markers are relatively high, it seems unlikely that DNA mismatch repair defects, resulting in increased MSI, are a major contributor to tumour genesis and progression in breast cancer.

The lack of MSI detection in our 66 early-onset breast cancer patients was unexpected. The collation of other microsatellite markers used to detect MSI in breast cancer [6] revealed marked variation in MSI detection rates for the same marker by different groups. For example, at the CA di-nucleotide marker DIS104, De Marchis and colleagues reported that six of 81 lymph-node-positive breast cancer patients were MSI positive [5], yet others had found no MSI at this marker [42]. It is possible that patient age, ethnic background, or tumour type impacts on MSI detection rates in breast cancer. All our samples were collected from women in South Australia who were at or under the age of 45 years at diagnosis, regardless of breast tumour type or ethnic group. Breast cancer patients

under 35 years of age have previously been studied for MSI [29], and detection rates of 5.6% (9/160 analyses) reported. This is slightly greater than the overall MSI detection rate observed in breast cancer (2.9%). Comparison of tumour type and patient demographics with other reports was difficult, as patient selection criteria were not always apparent.

Why then, is MSI not a major feature of breast cancer? It has been proposed that all malignant cells require some mechanism to acquire the large numbers of mutations required to achieve a growth advantage over normal cells [43, 44]. A tissue-specific gatekeeper gene is then inactivated and tumour progression escalates [45]. This inactivation results from a variety of mechanisms, the best described of which is the defective proofreading of DNA synthesis, facilitated by faulty DNA mismatch repair proteins, and detectable as MSI. Other mechanisms by which this may occur include elevated rates of abnormal chromosome segregation during mitosis, resulting in aneuploidy [46, 47] and widespread methylation and inactivation of important genes [48] such as those monitoring DNA mismatch repair [49]. The gatekeeper gene for breast cancer has yet to be identified, but it is possible to speculate that it may not contain sufficient microsatellite repeats to allow the mass perpetuation of mutations required for tumour progression. It is hoped that the database of breast cancer microsatellite markers assembled in conjunction with this study [6] will be a useful resource for those considering selection of microsatellite markers for investigations of the role of MMR defects in breast cancer in the future.

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Address for offprints and correspondence: D Quinn, School of Pharmacy and Medical Science, University of South Australia, City East Campus, Adelaide, South Australia 5000; *Tel.:* 618-8302 2310; *Fax:* 618-8302 2389 *Email:* D.Quinn@unisa.edu.au