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Microsatellite Instability and Loss of Heterozygosity in Primary Breast Tumours

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Abstract
Allelic imbalance or loss of heterozygosity (LOH) studies have been used extensively to identify regions on chromosomes that may contain putative tumour suppressor genes. We looked for evidence of microsatellite instability (MI) and LOH on chromosome 7q, 10q, 11p and 17q using seven polymorphic microsatellite markers. In 42 paired breast cancer-peripheral blood DNA samples we identified 24 tumours (57%) exhibiting genetic alterations. Twenty-one specimens exhibited LOH (50%), while 11 specimens exhibited MI (26%) in at least one microsatellite marker. The most frequent incidence of LOH was found for the marker THRA1 (8/33, 24%) indicating that *thra1* gene becomes a strong candidate tumour suppressor gene, whereas of MI it was D10S109 (3/26, 12%). These MI and LOH data were analysed using a range of clinicopathological parameters. Tumours displaying MI with no evidence of LOH and tumours exhibiting MI and LOH belonging to stage II or III were found, however none were at stage I. These data suggest that MI may be an early event in mammary tumorigenesis whereas LOH occurs at a late stage. A significant association between the absence of oestrogen receptors ($p < 0.01$) and the absence of both oestrogen and progesterone receptors ($p < 0.001$) at 17q21 were observed, indicating a possible relationship between specific genetic changes at this region and hormonal deregulation in the progression of breast cancer.

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Introduction

Breast cancer accounts for the most common cancer in women in Europe, currently affecting 1 in 12 women [1]. A subset of molecular alterations have been associated with the development of the disease. These alterations include the activation of proto-oncogenes, such as *ras* [2], and the inactivation of tumour suppressor genes (TSGs) such as *p53* [3]. Most of these genetic alterations are in the form of point mutations providing evidence for substitution mutagenesis as the major mechanism of damage to the genes. Recently, a second distinct type of genetic alteration based on misalignment mutagenesis has been documented in colorectal cancer [4–6]. This type of mutagenesis involves genomic DNA sequences termed 'microsatellites', in which 1–6 nucleotide motifs are tandemly repeated numerous times. Microsatellite instabillity (MI) has been related to defects in the DNA repair system and manifested through the alteration of DNA repeat size. Mutation of the microsatellite repeats originates as slippage owing to strand misalignment during DNA replication, without preference for contraction or expansion of the parental allele [7]. Although a number of reports exists on MI in various tumours, its real significance in tumour progression is unknown.

The inactivation of TSGs plays a critical role in multistage carcinogenesis [8]. The identification of novel TSGs provides information for the molecular pathway of cancer development. At present, loss of heterozygosity (LOH) using highly polymorphic microsatellite markers is the most common methodology employed for the localisation of sites in the genome with a high probability for the presence of candidate TSGs. A large number of studies exist on LOH in invasive breast cancer and virtually every human chromosome has been shown to exhibit allelic loss. Allelic losses at 1q, 3p, 6q, 7q, 9q, 11q, 15q, 16q, 17p, 17q, 18q and X have also been frequently found in breast cancer [9].

In the present study, we investigated the incidence of MI and LOH at 17q, 7q, 10q and 11p in 42 breast carcinomas, using a bank of seven highly polymorphic microsatellite markers. To determine the stage when MI and LOH may occur, we correlated the findings with clinicopathological parameters and we propose that these two events may take place in different stages during the progression of breast cancer.

Materials and Methods

Tumour Specimens and DNA Extraction

The breast tumour specimens were obtained from the 'H. Venizelou' Hospital and the 'Prolipsis' Medical Diagnostic Centre, Athens. Directly after dissection the specimens were stored at −70 °C until DNA extraction. A matched normal DNA control from blood was analysed. All the specimens corresponded to the primary tumours. DNA was extracted as previously described [10] and stored at 4 °C until PCR amplification.

PCR Amplification, Microsatellite and LOH Analysis

The DNA samples were examined for genetic alterations at seven different microsatellites by PCR amplification. The markers were selected on the basis of two criteria: (1) the ability to combine primers so that all seven loci could be studied in only two PCR reactions, and (2) different chromosomal locations, representing regions that show a variable degree of alterations in the genesis of these cancers. Two of the microsatellite markers lie proximal to *BRCA1* (D17S250, THRA1), D17S855 lies within the *BRCA1*, D17S579 lies distal to *BRCA1*, while the HRM marker is located in the first intron of H-*ras* proto-oncogene at 11p15.5. Markers D7S473 and D10S109 are located on chromosomes 7p12-q21 and 10q21, respectively.

PCR analyses were performed in a 50-μl reaction volume containing 200 ng of genomic DNA, 1 μ*M* of each primer, 250 μ*M* dNTPs, 5 μl of 10 × buffer (670 m*M* Tris·HCl, pH 8.5; 166 m*M* ammonium sulphate;

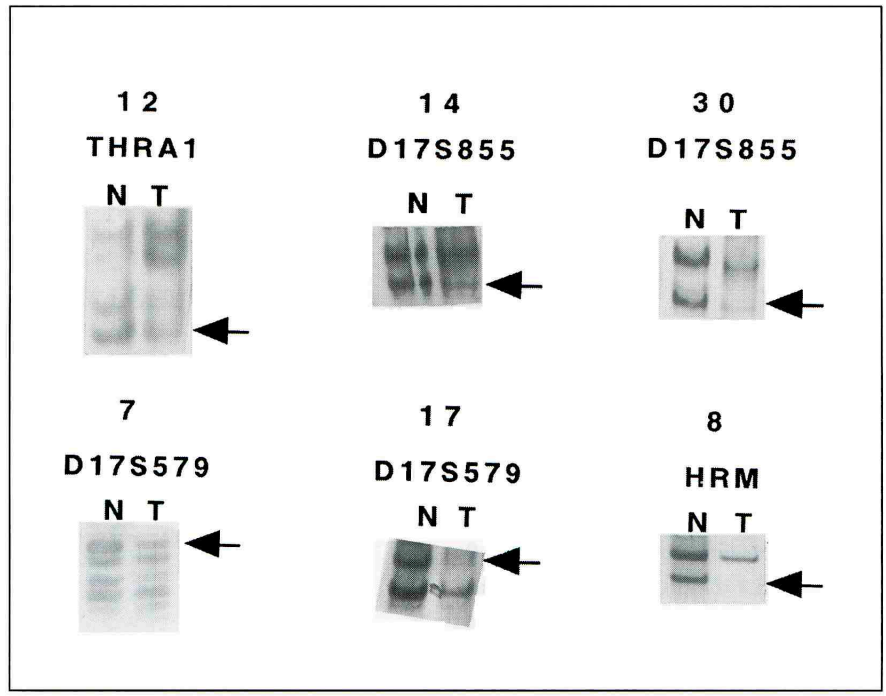

Fig. 1. Representative examples of LOH detected in breast cancers. N = Normal DNA; T = tumour DNA. Arrows indicate the position of a deleted allele. The faint bands in the position of the deleted alleles are interpreted as contamination by the adjacent normal tissue.

67 m*M* magnesium chloride; 1.7 mg/ml BSA; 100 μ*M* β-mercaptoethanol and 1% (w/v) Triton X-100) and 1 U of *Taq* DNA polymerase. Ten microlitres of the PCR product were analysed in a 7% polyacrylamide gel and silver-stained. The reactions were denatured for 5 min at 95°C and the DNA was subsequently amplified for 35 cycles at 95, 57 and 72°C each step. MI was scored by comparing the electrophoretic pattern of the microsatellite markers amplified from the paired DNA preparations that corresponded to the tumour versus the peripheral blood, demonstrating a shift of one or both of the alleles in the tumour DNA specimen. The shift was indicated by either an addition or deletion of one or more repeat units resulting in the generation of novel microsatellite alleles. For MI scoring all the heterozygous cases were counted as well as those that were constitutionally homozygous (non-informative) for a marker. The analysis in the MI-positive cases was repeated at least twice and the results were highly reproducible. Allelic losses were scored as significant decreases in intensity of one allele relative to the other as determined from comparison of tumour and normal DNAs, from individuals who were heterozygous for the given locus.

Statistical Analysis

All statistical analyses were performed using the χ^2 test. One-tailed p values <0.05 were considered statistically significant.

Results

Forty-two tumour specimens from patients with breast carcinomas were assessed for MI and LOH at 7q, 10q, 11p and 17q arms. Representative examples of specimens with LOH and MI are shown in figures 1 and 2, respectively. Twenty-four from 42 (57%) breast tumours exhibited genetic alterations (table 1). Twenty-one specimens exhibited LOH (50%), while 11 specimens exhibited MI (26%) in at least one microsatellite marker. No particular specimen exhibited a high incidence of MI, indicating the absence of a 'mutator phenotype', as reported in HNPCC [4]. The highest incidence of LOH was found for the marker THRA1 (8/33, 24%), while the highest one of MI was found for the marker D10S109 (3/26, 12%). The frequencies of incidences for all the microsatellite markers are shown in table 2. No samples with homozygous deletions of the region were found. Several tumours (i.e. 7, 14, 24 and 30) exhibited partial deletions of 17q which are probably due to multiple mitotic

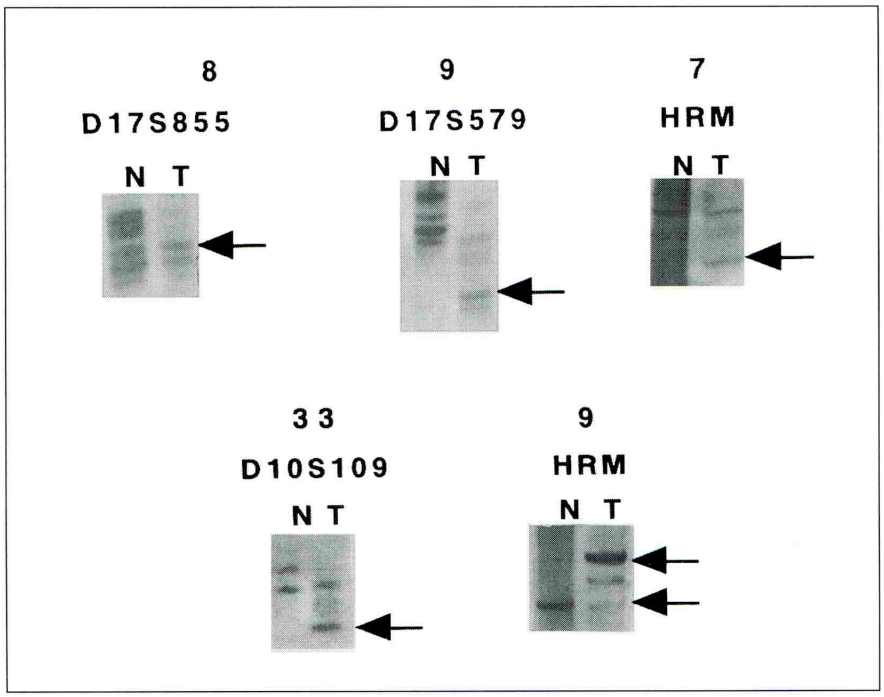

Fig. 2. Representative examples of specimens exhibiting MI. N = Normal DNA; T = tumour DNA. In all cases a shift in the mobility of the microsatellites is obvious and thus the specimens were scored as positive for MI. Patient 9 exhibited LOH and MI as indicated by the arrows; the first allele was deleted, while the remaining one showed an altered mobility.

recombination events (table 1). No sample exhibited deletions of all informative markers tested in chromosome 17, indicating a loss of the whole 17q arm. It is noteworthy that except for three specimens, the rest were non-informative for the marker HRM. There were also two specimens exhibiting LOH and MI simultaneously for different markers (No. 7 for D10S109 and No. 9 for HRM). These specimens appeared to have extended genetic alterations, concerning either LOH or MI in several markers at 17q.

The breast tumours studied here may be divided into three stages of disease as indicated in table 3. All tumours were grouped by stage and extent of LOH (no LOH, LOH for only one marker and LOH for two or more microsatellite markers). A similar comparison of disease stage with MI is presented in table 4. The distribution of tumour genomes showing instability of microsatellites in each disease stage is quite different from the data obtained for LOH. Of the 42 tumours, 27 showed no evidence of MI, whereas 9 tumours exhibited MI for one marker. Only 2 tumours displayed MI for two or more markers and both were stage II.

The LOH and MI results were studied in association with other clinicopathological parameters apart from the stage of the disease, but no correlation was found with nodes at pathology, age (on average 58 years old, range 33–92), site or with the histological differentiation of the tumours. However, the number of tumours between 1 and 2 cm size showing evidence of LOH and MI at 17q was significantly less (5/16 and 4/16, respectively) compared with the respective tumours having the same depth of tumour invasion, indicating that possibly this particular alteration occurs in only a small fraction of cells and so the tumours seem to be heterogeneous at the early stage of the disease. This finding should be interpreted with caution, since the number of tumours with this size was small.

Furthermore, we looked for correlations between the genetic alterations and oestrogen (ER)/progesterone (PgR) receptor status. Re-

Table 1. Genomic instability in 42 breast tumours tested with 7 microsatellite markers

Patient	Microsatellite markers							Stage/ pTNM
	D17S250	THRA1	D17S855	D17S579	D10S109	HRM	D7S473	
1	–	H	–	H	–	N	–	$I/T_1N_0M_0$
2	–	N	–	–	–	N	–	–
3	–	LOH	–	–	–	N	–	$II/T_2N_1M_0$
4	–	N	–	LOH	–	N	–	$I/T_1N_0M_0$
5	H	–	H	H	H	N	H	–
6	H	H	H	LOH	H	N	H	$II/T_2N_1M_0$
7	N	H	LOH	LOH	LOH + MI	MI	H	$II/T_2N_1M_0$
8	LOH	H	H	MI	–	LOH	–	$III/T_3N_2M_0$
9	H	N	MI	MI	–	LOH + MI	–	$II/T_2N_1M_0$
10	LOH	MI	LOH	H	H	N	–	$II/T_2N_0M_0$
11	H	H	H	H	H	N	N	$II/T_3N_0M_0$
12	–	LOH	H	H	–	N	N	$III/T_3N_2M_0$
13	H	N	LOH	–	LOH	N	H	$II/T_2N_1M_0$
14	H	H	LOH	LOH	–	N	MI	$III/T_4N_2M_0$
15	H	H	MI	H	N	N	LOH	$II/T_2N_1M_0$
16	–	H	H	H	–	N	H	$II/T_2N_0M_0$
17	N	H	H	LOH	–	N	H	$III/T_2N_2M_0$
18	H	H	–	H	H	N	H	$II/T_2N_1M_0$
19	N	H	–	H	H	N	–	$III/T_3N_2M_0$
20	H	H	H	H	H	N	–	$II/T_1N_1M_0$
21	H	LOH	H	H	H	N	H	$III/T_4N_3M_0$
22	MI	N	H	H	H	N	H	$I/T_1N_0M_0$
23	N	H	–	H	LOH	N	H	$II/T_2N_1M_0$
24	LOH	LOH	–	MI	H	N	LOH	$II/T_2N_0M_0$
25	H	H	H	–	H	N	–	$II/T_2N_0M_0$
26	–	H	–	–	H	N	MI	$II/T_2N_1M_0$
27	H	N	–	–	–	N	–	$II/T_1N_1M_0$
28	–	LOH	N	H	H	N	H	$II/T_2N_1M_0$
29	–	LOH	–	–	H	N	N	$II/T_2N_1M_0$
30	–	H	LOH	LOH	–	N	N	$II/T_2N_1M_0$
31	–	N	–	–	H	N	–	$II/T_1N_1M_0$
32	H	LOH	H	H	H	N	H	$III/T_4N_3M_0$
33	H	H	H	H	MI	N	H	$I/T_1N_0M_0$
34	H	H	H	H	H	N	H	$III/T_4N_0M_0$
35	–	N	H	H	H	N	LOH	$I/T_1N_0M_0$
36	H	LOH	H	H	MI	N	H	$II/T_2N_0M_0$
37	H	H	H	H	H	N	H	–
38	H	H	H	H	–	N	H	$I/T_1N_0M_0$
39	H	H	–	–	–	N	H	–
40	H	H	H	–	H	N	H	$III/T_3N_3M_0$
41	H	H	H	H	–	N	H	$I/T_1N_0M_0$
42	H	H	H	–	–	N	H	–

H = Heterozygosity; N = non-informative; – = no data.

Table 2. LOH and MI in breast carcinomas

Microsatellite marker	Chromosomal localisation	LOH	MI
D7S473	7p12-q21	3/26	2/30
D10S109	10q21	3/25	3/26
HRM	11p15.5	2/3	2/42
D17S250	17q11.2-q12	3/26	1/30
THRA1	17q11.2-q12	8/33	1/38
D17S855	17q21	5/28	2/29
D17S579	17q21	6/31	3/31

Table 3. Tumour stage and number of informative cases displaying LOH

Stage	Number of informative cases with LOH		
	0	1	≥2
I	5	1	0
II	8	9	5
III	3	4	2

Table 4. Tumour stage and number of informative cases displaying allelic imbalances

Stage	Number of informative cases with MI		
	0	1	≥2
I	4	2	0
II	15	5	2
III	7	2	0

sults are shown in tables 5 and 6. ER-negative status was more frequent in tumours that exhibited genetic alterations at 17q21 (10/12, 83%) than in tumours that retained both alleles (6/22, 27%). Similarly, PgR-negative status was more frequent in tumours that displayed allelic imbalance at 17q21 (8/12, 67%) than in tumours that retained both alleles (9/22, 41%). The negative status for both ER and PgR was more frequent in tumours with LOH and MI at 17q21 (8/9, 89%) than in tumours which retained both alleles (2/14, 14%). In contrast, no correlation was found between genetic alterations and ER/PgR receptor status at 17q11.2-q12.

Discussion

In the present study we analysed the incidence of MI and LOH at four chromosomes in a set of breast tumours. A significant incidence of LOH was found for the 17q arm suggesting that important TSG(s) for the development of breast cancer may be located on this chromosomal region. Deletions at 17q occur frequently in a variety of neoplasms. These include ovarian tumours (flanked by THRA1 and D17S75) [11], oesophagus tumors between probe C117-316 and C117-710 [12], laryngeal tumours between D17S250 and D17S579 [13], non-small-cell lung [14] and prostate [15] which are also associated with deletions at 17q near the *BRCA1* region. The wide spectrum of human cancers affected by alterations of the candidate TSG(s) of 17q suggests a significant role for these genes in the development of the neoplasia.

Forty-one percent of the breast tumours tested exhibited allelic deletions at 17q. Marker D17S250 exhibited an LOH incidence of 12% (3/26) which is probably due to the background LOH incidence of the tumours tested.

BRCA1 has been considered a TSG [16], in which deletions at 17q are targeted. However, recent reports cast doubt on the role of *BRCA1* as the main tumour suppressor of the 17q arm, an argument supported by the re-

Table 5. Correlation between genetic alterations and ER/PgR receptor status

Genetic alteration	ER			PgR		
	–	+	p value	–	+	p value
17q11.2-q12						
LOH and MI	6	6		6	5	
Retain	11	15	NS	13	13	NS
17q21						
LOH and MI	10	2		8	4	
Retain	6	16	$p < 0.01$	9	13	NS

ER – or PgR – represents ER or PgR receptor level below 5 fmol mg^{-1} protein, ER + or PgR + ER or PgR receptor level above 5 fmol mg^{-1} protein. NS = Not significant.

Table 6. Correlation between genetic alterations and concordant ER/PgR receptor status

Genetic alteration	Both –	Both +	Statistical significance
17q11.2-q12			
LOH and MI	6	4	
Retain	6	10	NS
17q21			
LOH and MI	8	1	
Retain	2	12	$p < 0.001$

ER – or PgR – represents ER or PgR receptor level below 5 fmol mg^{-1} protein, ER + or PgR + ER or PgR receptor level above 5 fmol mg^{-1} protein. NS = Not significant.

sults of the present investigation. *BRCA1* is linked to the microsatellite marker D17S855 and is located between the markers D17S250 and D17S579 [17]. Although we found an LOH incidence of 12 and 19% for these markers, respectively, the LOH incidence of the THRA1 marker was higher (24%). This marker is closely linked, but distinct from the markers D17S855 and D17S579. High percentages of LOH for the THRA1 marker have been reported previously [18]. Based on the hypothesis that the sporadic breast cancer gene is allelic with the *BRCA1* locus, the *thra1* gene or other genes located proximal to the THRA1 locus become strong candidate TSGs. The potential role of *thra1* as a TSG has been reviewed [19]. Thus, we found it reasonable to postulate that *BRCA1* is not the direct target of the deletions, but gene(s) at the near vicinity of *BRCA1* are the main TSG(s) of this chromosomal area. Recent data suggest that *BRCA1* is a member of a family of genes that function as tumour suppressors. The q arm of the human chromosome 17 has been described to harbour TSG(s) with an effect in various human tumours including breast cancer [20–22].

LOH incidence was 12% (3/25) for the marker D10S109. This is the first report to our knowledge on allelic losses at 10q21 in breast cancer. There are two reports on studies examining this locus with marker D10S109, but no case with LOH or MI was found [23, 24]. As regards the HRM marker located within the first intron of the H-*ras* proto-oncogene, the heterozygosity was low. However, all three informative cases displayed MI or LOH, indicating that the repeti-

tive elements within the H-*ras* gene are a possible target for genetic aberrations.

The significant and consistent alterations in the microsatellite markers in breast tumours suggest that genomic instability has occurred in these tumours. MI has been reported not only for breast cancer [25, 26] but also in several other tumours [4, 6, 27, 28]. Our results provide important evidence for genomic instability at loci on chromosomes 7q, 10q, 11p and 17q indicating that MI is a detectable phenomenon in breast cancer. As regards the clinicopathological parameters, the occurrence of microsatellite alterations in tumours was independent of age, type, site and grade of primary carcinomas.

Because LOH is such a frequent event in breast cancer, we investigated its relationship to the genetic MI. None of the stage I tumours exhibiting MI showed evidence of LOH. Clearly stage II breast cancers that displayed considerable LOH and microsatellite instability cannot give rise to stage III tumours with no apparent LOH or MI (tables 3, 4). All cases affected with MI and LOH concurrently belonged to stages II or III. We propose, therefore, that there are two different mechanisms underlying the progression of breast cancer, one that proceeds from an early event involving overt loss or gain of a repetitive unit in the microsatellite DNA and another that may involve point mutations or small deletions. Mutations in DNA repair genes are known to cause genomic instability in both meiotic and mitotic cells of *Drosophila* [29]. Furthermore, as MI was found in all the specimens from patients with multiple colorectal tumours and adenomas, it was proposed that a mutation had occurred in the DNA repair gene [30]. MI has now been correlated with mutations in the *hMSH2, hMLH1, hPMS1* and *hPMS2* genes which have homology to bacterial and yeast genes participating in mismatch repair [4, 30, 31]. Moreover, mutations in these mismatch repair genes have been linked to loci on chromosomes 2p15-16, 3p21-23, 2q31-33 and 7p22 (*hMSH2, hMLH1, hPMS1* and *hPMS2*) in hereditary nonpolyposis colorectal cancer (Lynch syndrome) [31]. It would be of interest to screen DNA repair genes for mutations in breast cancers exhibiting MI and investigate whether these mutations are also present in the germline of the patients. The latter may provide clues for the hereditary basis of the disease and is consistent with the observation that DNA repair deficiency may occur in phenotypically normal cells [32].

Genetic alterations at 17q21 were significantly associated with an ER- and PgR-negative state, whereas we did not detect an association between LOH and MI at 17q11.1-q12 and these hormone receptors. Our results are consistent with a previous study [33], reporting an association of genetic alterations at 17q21 and loss of hormone receptors in breast cancer, whereas no significant correlation was detected between them at 17q13.3. Normal breast epithelial cells and early-stage breast cancer cells are under the control of ER and PgR, but only a quarter of breast cancers retaining heterozygosity show ER and PgR dependency. The mechanism of this loss of hormone dependency in breast carcinogenesis is obscure. An association of the loss of hormone receptors with specific genetic alterations at 17q21 implies that alterations of some gene(s) at this region might have some relationship to events that render cancer cells independent of hormonal control.

The frequency of the LOH occurrence in breast cancer as well as the finding that genetic MI is a detectable phenomenon in breast cancer made us investigate the relationship between them. Tumours displaying MI with no evidence of LOH and tumours exhibiting MI and LOH belonging to stage II or III were found, however none were at stage I. These data suggest that MI may be an early event in mammary tumorigenesis whereas LOH oc-

curs at a later stage. This is consistent with other reports [26, 34].

Considering the role of MI in tumorigenesis, it is noteworthy that MI in other cancers like urinary bladder cancer or colorectal cancer occurs in low stage tumours [35] or is associated with a good prognosis [6]. To determine if these changes in the microsatellite repeats constitute prognostic indicators could be of importance.

The precise mechanism through which MI contributes to tumorigenesis is unknown. Microsatellites are scattered throughout the genome including non-coding and coding regions of various genes. It is conceivable that MI adversely affects the functional activity of such genes. Studies focusing on the onset of such genetic events and the mechanism of observed genomic instability in breast carcinomas are necessary.

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