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Loss of Heterozygosity and Microsatellite Instability at *RAD52* and *RAD54* Loci in Breast Cancer*

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This study was carried out to evaluate the loss of heterozygosity (LOH) and microsatellite instability (MSI) in breast cancer, in the 12p13.3 and 1p32 chromosomal regions where *RAD52* and *RAD54* genes are localized. Polymorphic markers D12S98, D12S1698 for *RAD52* and D1S209, D1S411 for *RAD54* were used. Relationships between LOH and clinicopathological parameters, i.e. tumor type and grade, patient's age, steroid receptors status and lymph node and distal metastases were assessed. For alleles frequency estimation 100 primary breast cancers were tested. DNA isolated from paraffin-embedded tissues and their matched blood samples were analyzed for PCR-based LOH and MSI by fluorescence-based DNA sequencing technology. In analyzed cases LOH was found in 14% and 11% of informative cases for D12S98 and D12S1698 markers, respectively and in 18% and 17% of informative cases for D1S209 and D1S411 markers, respectively. The highest frequency of MSI was identified at loci D12S98 (10%) and D1S209 (11%). Significant correlations between *RAD52* and *RAD54* regions with concomitant LOH and histological type and progesterone receptor status were observed. In the case of *RAD54* further correlations with respect to tumor grade and the presence of distal metastases were noticed.

Introduction

One of the characteristics of malignant cells is the widespread instability of their genome. Excessive proliferation activity and increased mutation rate both contribute to the genomic dissociation of cancer cells. Allelic imbalance studies constitute one of the main investigative tools for detecting genetic alterations in oncology research. For a given somatic genetic *locus*, mammalian cells carry two alleles: one of paternal and one of maternal origin. Detection of dissimilar amounts of DNA between the two alleles in neoplastic cells, compared to normal cells, signifies allelic imbalance for the tumor studied. Most of the loci, however, contain genes that are not yet clearly implicated in the formation and/or progression of breast neoplasias [23, 28].

The repair of double-strand breaks in chromosomal DNA is of critical importance for the maintenance of genomic integrity. Double-strand DNA (dsDNA) damage may be repaired by homologous recombination, by the action of recombination and by repair proteins. In mammals, homologous recombination appears to be indispensable for cell viability and tumor suppression [10]. Homologous recombination in eukaryotic organisms is conserved in mechanism and mediated by group of proteins known as the RAD52. The RAD52 group members were first identified in *Saccharomyces cerevisiae*, and among these proteins RAD51, RAD52 and RAD54 play a crucial role in human cells [34].

Human RAD51 protein is a homologue of RecA protein of *Escherichia coli*. Its gene maps to chromosome 15q15.1 and encodes a putative 339-amino acid protein. It is transcribed at high levels in thymus, spleen, testis and ovary [42]. RAD51 protein binds to single and double-strand DNA, exhibits DNA-dependent ATPase activity and forms nucleoprotein filaments with DNA [2, 6, 15, 29].

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Human RAD52 protein is not required for the initiation of recombination, but is essential for the intermediate stage following the formation of double-strand breaks but before the appearance of stable recombinants [35]. Its gene maps to chromosomal region 12p13.3, and encodes a 418-amino acid protein [31].

Human RAD54 protein belongs to a superfamily of DNA helicases that unwind duplex DNA forming single-strand DNA (ssDNA), thus making them available for replication, recombination and repair [25]. Its gene maps to chromosomal region 1p32 and encodes a 747-amino acid protein [38].

After DNA double-strand break formation, the DNA ends at break sites are promptly processed by nucleases. The resulting 3'OH ending tails are then presumably coated by replication protein A (RPA), the eukaryotic single-strand-binding protein [7]. With the aid of accessory factors, RAD52 protein and RAD55-RAD57 complex, RAD51 displaces RPA from ssDNA tails and assembles into a nucleoprotein filament. This filament, also named presynaptic complex, is the molecular device for homology search on partner duplex DNA molecules and subsequent DNA strand exchange. Further steps comprise hybrid DNA extension, priming of DNA synthesis, branch migration and resulting of Holliday junctions, yielding DNA molecules repaired in an intrinsically error-free way [22].

The RAD52 and RAD54 proteins serve as accessory factors in RAD51-mediated joint molecule formation. The details of the molecular mechanisms through which RAD52 and RAD54 stimulate joint molecule formation are not well understood. RAD52 has been shown to increase the rate of annealing of complementary single strand molecules, to bind to DNA ends, to stimulate homologous pairing by RAD51, and to overcome the inhibitory effect of the ssDNA-binding protein replication protein A on RAD51 nucleoprotein filament formation [5, 30, 32, 43, 44, 47]. RAD54 was shown to interact with RAD51 [9, 12, 18, 46] and to possess ATPase activity. Importantly, the ATPase activity of RAD54 specifically requires the presence of dsDNA [37, 45]. This cofactor specificity is opposite to that of RAD51 [2]. Because the initial substrate of RAD51 during homologous recombination is ssDNA [3], it is likely that the substrate for RAD54 is the double-strand homologous repair template.

Polymorphism in multiple pathways may act synergistically with environmental carcinogen damage to increase cancer susceptibility [39]. High rate of loss of heterozygosity in the chromosomal region *RAD51* (15q15.1) has been suggested for breast cancer [13, Nowacka-Zawisza et al., in preparation]. Gonzalez et al. [13] observed also LOH in 12p13.3 and 1p32 chromosomal regions in 16% and 20% of breast cancer, respectively. The aim of the work was to verify if allelic losses in the chromosomal regions of *RAD52* and *RAD54* genes could be notoriously found in breast cancer. Relation of these molecular alterations with clinicopathological features of the breast cancer was analyzed.

Materials and Methods

Patients

A hundred paraffin-embedded tissues from patients with breast cancer and matched blood samples were obtained at the Department of Clinical Pathomorphology, Polish Mother's Memorial Hospital Research Institute, Łodź, Poland. The median age of patients was 53, with range of 28–77. Among patients 34 were under or at the age of 50 and 66 were above the age of 50. All tumor specimens underwent clinicohistopathological evaluations. Regarding histological type 86 cases were classified as ductal and 14 cases were classified as lobular carcinoma. The series included 14 cases of grade I, 82 of grade II and 4 of grade III according to the modified Bloom-Richardson criteria; 62 positive and 38 negative cases in respect to estrogen receptors; 57 positive and 43 negative cases in respect to progesterone receptors. According to tumor size, the samples were divided into $T_1 \leq 2$ cm (64 cases), T_2 2–5 cm (31 cases) and $T_3 > 5$ cm (5 cases) categories [19]. According to the presence of metastases in the axillary lymph nodes, the samples were allocated to N_0 (63 cases), N_1 (32 cases) and N_2 (4 cases) categories. In 97 studied cases there were no distal metastases (M_0) and 3 cases were classified as M_1.

DNA isolation

DNA was isolated from peripheral blood and tissue samples following standard phenolic procedures. The paraffin-embedded tissue samples were extracted with xylene to remove the paraffin [40, 49].

PCR conditions and primers

Four microsatellite markers D12S98, D12S1698 and D1S209, D1S411 flanking dinucleotide CA repeats at the 12p13.3 and 1p32 region, respectively were used to determine LOH and MSI for *RAD52* and *RAD54* genes in breast cancer. Sequences for primers are listed in the Human Genome Database. Primers were synthesized and labeled fluorescently at Applied Biosystems (USA). The DNA sequences of primers (5'→3') were as follows: D12S98 – ATT GGG GAC CCG TGC TA, CAA AGC CTG ACG TAG AAG CATT; D12S1698 – ATA GCC TAT CAT GGG ACT TTG CTTT, GTT TTC CAG AGG GCC AGA ACT AAT; D1S209 –

AAA CAT CAG TGT TCC ATC ATA GAC, AGC TTT GGG GGA CAT AAC AT; D1S411 – GAG GTC AGT TGA TCC AGT GG, AAG GTT TCT GAG AAC TTT TTG TG. Polymerase chain reaction (PCR) was performed in 7.5 μl volumes using 50 ng of genomic DNA template, 0.3 units of AmpliTaq Gold^{TM}DNA polymerase (5 U/μl), 1 × GeneAmp® PCR Gold Buffer (10 × concentration), 1 mM GeneAmp dNTP Mix (10 mM), 2.5 mM magnesium chloride (25 mM), 5 pmol of either forward or reverse primer end labeled with the dye phosforamidite 6-FAM and TET (Applied Biosystems, USA). 30-cycles amplification (denaturation, annealing, extension) was done in GeneAmp 2400 PCR System (Perkin-Elmer, USA). Profile times and temperatures were: 95°C for 5 min; 30 cycles – 94°C for 15 s, 55°C for 30 s, 72°C for 30 s; 72°C for 10 min.

LOH and MSI analysis

Samples of 3 μl PCR products were added to 4 μl of a master mix containing ten parts of deionized formamide, two parts of GeneScan™-350 AMRA Size Standard and one part of loading buffer (50 mg/ml blue dextran, 25 mM EDTA). Samples were denatured by heating at 95°C for 5 min and chilled on ice. The 3 μl of each sample were loaded onto 5% Long Ranger (FCM® BioProducts, USA) gels with 6 M urea and 1 × TBE (10 × TBE: 0.89 M Tris borate, 0.02 M EDTA, pH 8.0) according to the standard protocol. Fluorescent PCR products were analyzed in ABI PRISM 377™ DNA Sequencer. The fluorescent signals from different size alleles were recorded and analyzed using GeneScan version 3.1.2 software and Genotyper version 2.5 (Applied Biosystems, USA).

Statistical analysis

All comparisons between LOH and clinicopathological parameters were performed using χ^2 test with the Yates correction. One-tailed *P*-values of ≤0.05 were considered statistically significant. Statistical analysis was performed using the STATISTICA package, version 5.

Results

LOH and MSI at the 12p13.3 and 1p32 regions in breast cancer

The LOH analysis in 12p13.3 and 1p32 chromosomal regions was performed for microsatellite markers D12S98, D12S1698 in the case of *RAD52* and D1S209, D1S411 in the case of *RAD54*. DNA was isolated from the tumor sample and corresponding peripheral blood of each of 100 patients. The results for representative cases are shown in Table 1.

TABLE 1
LOH and MSI for representative breast cancer cases

No. of patient	*RAD52*		*RAD54*	
	MICROSATELLITE MARKER			
	D12S98	D12S1698	D1S209	D1S411
1.	❑	❑	N	●
2.	❑	N	■	N
4.	❑	●	❑	N
5.	N	❑	●	❑
6.	❑	❑	❑	●
7.	❑	N	●	❑
8.	●	❑	❑	N
10.	■	N	❑	N
11.	❑	●	❑	❑
13.	N	N	N	■
14.	❑	❑	●	❑
15.	❑	❑	❑	N
16.	●	❑	❑	❑
18.	■	❑	❑	■
19.	N	❑	❑	●
20.	❑	●	N	❑
21.	N	N	●	❑
22.	❑	■	❑	❑
24.	●	N	■	❑
25.	N	❑	N	●
27.	❑	❑	●	❑
28.	●	❑	N	N
30.	N	■	❑	❑
31.	❑	●	❑	❑
32.	❑	❑	●	❑
33.	N	N	■	N
34.	●	❑	N	❑
36.	N	❑	❑	●
37.	❑	■	❑	❑
38.	N	N	●	❑
40.	■	❑	N	❑
41.	❑	●	❑	❑
42.	❑	❑	■	❑
43.	N	❑	❑	●
44.	●	❑	❑	❑
45.	N	❑	●	❑
47.	❑	N	❑	■
49.	■	❑	❑	❑

50.	N	N	■	N
52.	❑	●	❑	❑
53.	N	N	❑	●
56.	❑	❑	❑	■
57.	●	❑	N	❑
58.	❑	N	●	N
59.	❑	■	❑	❑
60.	❑	❑	❑	●
61.	❑	●	❑	❑
64.	■	❑	■	❑
65.	❑	❑	●	❑
66.	❑	■	❑	❑
67.	❑	❑	❑	●
69.	❑	❑	■	N
70.	●	❑	❑	■
71.	❑	❑	●	❑
73.	■	❑	❑	N
75.	❑	❑	❑	●
81.	●	N	●	❑
84.	❑	❑	●	❑
85.	■	❑	❑	❑
86.	❑	●	N	❑
88.	N	❑	■	N
89.	❑	■	❑	●
90.	●	❑	N	❑
91.	❑	❑	❑	●
92.	N	●	❑	N
93.	■	❑	●	❑
94.	❑	N	❑	❑
95.	❑	❑	■	❑
96.	●	❑	❑	N
98.	❑	❑	❑	●
99.	❑	❑	❑	■
100.	N	❑	●	N

● LOH; ■ MSI; ❑ heterozygous with no loss; N not informative

Genomic deletion detected by allelic loss was observed in 14% (11/78) and 11% (9/82) of informative cases for D12S98 and D12S1698 markers, respectively and in 18% (15/84) and 17% (13/78) of informative cases for D1S209 and D1S411 markers, respectively (Table 2).

Microsatellite instability was noted for marker D12S98 in 10% (8/78), D12S1698 in 7% (6/82), D1S209 in 11% (9/84) and D1S411 in 8% (6/78) of cases that were informative (Table 2).

TABLE 2
LOH and MSI analysis in *RAD52* and *RAD54* chromosomal regions in breast cancer

Gene Chromosomal localization	Microsatellite marker	Number of informative cases	Tumors	
			with LOH (%)	with MSI (%)
RAD52 12p13.3	D12S98	78	11 (14)	8 (10)
	D12S1698	82	9 (11)	6 (7)
RAD54 1p32	D1S209	84	15 (18)	9 (11)
	D1S411	78	13 (17)	6 (8)

LOH in RAD52 and RAD54 chromosomal regions and clinicopathological status correlation

LOH in the 12p13.3 and 1p32 chromosomal regions and clinicopathological parameters were compared using χ^2 test with the Yates correction. Differences between the two populations were judged significant at confidence levels greater than 95% ($P<0.05$). Significant correlations between *RAD52* and *RAD54* regions with concomitant LOH and histological type and progesterone receptor status were observed. In the case of *RAD54* further correlations with respect to tumor grade according to Bloom-Richardson classification and the presence of distal metastases were found (Table 3).

Discussion

Breast cancer is the most frequent malignancy in women. It is also the most common cancer-related cause of death among women [36]. High prevalence and mortality of breast carcinoma underscore the necessity for solid clarification of the molecular basis of the disease. LOH has been extensively studied in breast cancer, as it constitutes one of the commonest genetic alterations in this type of cancer [33]. Identification of chromosomal regions with allelic losses is a useful method for screening genes implicated in the breast pathogenesis and, at the same time, offers the opportunity to investigate new parameters with possibly high sensitivity and specificity for use as prognostic factors [11].

Mutations in *RAD51*, *RAD52* and *RAD54* genes in human tumors have been reported. Kato et al. [21] screened Japanese patients with hereditary breast cancer for *RAD51* mutations and found single alteration in exon 6: a G-to-A transition converting codon 150 from CGG (Arg) to CAG (Gln). Both diagnosed patients had bilateral breast cancer,

TABLE 3
Relationship between LOH in the *RAD52* and *RAD54* regions and clinicopathological parameters of breast cancer

Characteristics	N	*RAD52*						*RAD54*					
		MICROSATELLITE MARKER											
		D12S98			**D12S1698**			**D1S209**			**D1S411**		
		I	LOH	*P*	I	LOH	*P*	I	LOH	*P*	I	LOH	*P*
Tumors	100	78	11	—	82	9	—	84	15	—	78	13	—
Age													
≤ 50	34	34	6	NS	25	5	NS	32	9	NS	35	8	NS
> 50	66	44	5		57	4		52	6		43	5	
Histologic type													
ductal carcinoma	86	69	7	**0.02**	77	6	**0.01**	77	11	**0.02**	67	8	**0.02**
lobular carcinoma	14	9	4		5	3		7	4		11	5	
Grade (modified Bloom-Richardson criteria)													
I	14	10	1	NS	9	2	NS	10	5	**0.02**	8	4	**0.02**
II	82	65	9		71	7		71	10		69	9	
III	4	3	1		2	—		3	—		1	—	
Tumor size (cm)													
T1	64	49	7	NS	59	4	NS	55	8	NS	53	6	NS
T2	31	27	3		21	3		27	7		21	4	
T3	5	2	1		2	2		2	—		4	3	
Axillary lymph node status													
N0	63	41	4	NS	54	4	NS	62	7	NS	57	7	NS
N1	32	33	4		27	4		19	6		19	6	
N2	4	4	3		1	1		3	2		2	—	
Metastases													
M0	97	76	10	NS	80	8	NS	82	13	**0.01**	75	10	**0.01**
M1	3	2	1		2	1		2	2		3	3	
Estrogen receptors													
Positive	62	46	7	NS	59	6	NS	55	8	NS	48	9	NS
Negative	38	32	4		23	3		29	7		30	4	
Progesterone receptors													
Positive	57	47	3	**0.04**	23	6	**0.02**	53	5	**0.02**	44	11	**0.03**
Negative	43	31	8		59	3		31	10		34	2	

N total number of tumors studied; I informative cases; *P* χ^2 test with the Yeates correction

one with synchronous bilateral breast cancer and the other with synchronous bilateral multiple breast cancer. The patients were presumed to be unrelated. Two single base polymorphisms have been also identified in the 5'-untranslated region of *RAD51*: 5'UTR G135C and 5'UTR G172T [48]. *RAD51*-135C itself has not been demonstrated to lead to elevated risk of breast cancer or to be predictive of survival of breast cancer patients [8, 14]. However, *RAD51*-135C has been found to be associated with an elevated risk of breast cancer in *BRCA2* mutation carriers [20, 24]. Wang et al. [48] identified the *RAD51*-135C variant in slightly more *BRCA1* mutation carriers affected with breast cancer as compared to healthy carriers. Matched case study of Polish women showed instead that *RAD51*-135C is associated with decreased risk of breast cancer in women who carry the *BRCA1* mutation 5382insC [17]. No association

was detected between epithelial ovarian cancer risk and *RAD51*-G135C and *RAD51*-G172T [1]. On the other hand both *de novo* and therapy-related acute myeloid leukemia (AML and t-AML) seem to be associated with the *RAD51*-135C polymorphism [41].

Bell et al. [4] and Han et al. [16] have indicated no association between a stop codon polymorphism (Ser346ter) in *RAD52* and breast cancer risk.

Matsuda et al. [26] have described missense mutations at functional regions of *RAD54* and the absence of the wild type *RAD54* expression resulting from aberrant splicing in primary cancers. They observed Pro63-to-His substitution of the *RAD54* gene in colon adenocarcinoma and Val444-to-Glu substitution in non-Hodgkin lymphoma. Although Pro at codon 63 and Val at codon 444 are outside helicase motifs, Matsuda et al. [26] suggest that these amino acid substitutions affect the function of *RAD54*. In one of 95 breast cancers studied, Matsuda et al. [26] found G-to-A transition in the *RAD54* gene converting Gly to Arg at codon 325 within helicase motif III (AccIII site). The absence of the wild type allele indicated that the tumor was homozygous (or hemizygous) for the mutation. The corresponding normal tissue showed the same transition, indicating that this was a germline mutation. A restriction-based screen, developed from the fact that the Gly325-to-Arg mutation abolished AccIII site, revealed that this mutation was absent in 100 normal individuals. The patient in the particular one case was a 63-year-old woman with no obvious family history of cancer.

High frequency of chromosomal deletions elicited as loss of heterozygosity in several chromosomal regions is a hallmark of genomic instability in breast cancer [27]. In the case of the *RAD52* epistasis group in breast cancer Gonzalez et al. [13] observed allelic loss for at least one marker in 31 (32%) of the 98 cases that were informative for microsatellite of the 15q15.1 region; in 14 (16%) of the 90 cases that were informative for microsatellite of the 12p13.3 region and in 22 (20%) of the 109 cases that were informative for microsatellite of the 1p32 region. In our previous studies LOH was found in 46%, 41% and 29% of informative cases for microsatellite markers D15S118, D15S214 and D15S1006, respectively of the 15q15.1 chromosomal region in breast cancer [Nowacka-Zawisza et al., in preparation]. The data presented in this paper show genetic deletion detected by allelic loss in 14% and 11% of informative cases for D12S98 and D12S1698 markers, respectively and in 18% and 17% of informative cases for D1S209 and D1S411 markers, respectively. The significant rate of LOH in chromosomal regions of *RAD51*, *RAD52* and *RAD54* genes seems to indicate, that the genes of these regions may play a role in the development of breast cancer and in determining the pathologic features of the tumors. Especially those significant correlations between *RAD51*, *RAD52* and *RAD54* regions with concomitant LOH and clinicopathological parameters of breast cancer were observed. In the case of *RAD51* chromosomal region this correlation includes estrogen and progesterone receptor content, as well as tumor grade and stage [13, Nowacka-Zawisza et al., in preparation]. Similarly, in the case of *RAD52* and *RAD54* chromosomal regions, it includes progesterone receptor status, as well as tumor grade and stage. These data suggest that the genes of these regions may influence the specific pathological phenotype of breast carcinomas. They may perform common or synergistic functions in the control and progression of tumor cells, participating in these processes through the repair of dsDNA and normal chromosomal recombination mechanisms.

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