Molecular Medicine 4: 807-822, 1998

Molecular Medicine

Alterations of p16-pRb Pathway and Chromosome Locus 9p21-22 in Sporadic Invasive Breast Carcinomas

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Accepted November 3, 1998.

Abstract

The p16-pRb pathway represents a vital cell-cycle checkpoint. In the present study we investigated the alterations of this G1-phase protein pathway using immunohistochemical and molecular methods in a series of 55 breast carcinomas and correlated the findings with clinicopathological features of the patients. Furthermore, we examined its relationship with the status of the chromosomal region 9p21-22 performing a deletion map analysis because there are indications that, in addition to *CDKN2* and *MTS2/p15^{INK4B}* tumor suppressor genes (TSGs), this area harbors other TSG(s).

Aberrant expression (Ab) of p16 and pRb was observed in 26 (47%) and 16 (29%) of the carcinomas, respectively. A statistical trend pointing out an inverse relationship between p16 and pRb expression was found ($p = 0.079$). Analysis of the region that encodes for p16 by deletion mapping, a PCR-based methylation assay and PCR-SSCP, revealed that deletions and transcriptional silencing by methylation might represent the main mechanisms of *CDKN2/p16^{INK4A}* inactivation in breast carcinomas. The results of deletion mapping also suggest that another TSG(s) may reside at the 9p21-22 area particularly at the *D9S162* loci and that co-deletion of this putative gene with *CDKN2/p16^{INK4A}* may play a role in breast carcinogenesis. In addition, microsatellite instability (MI), a marker of replication error phenotype (RER+), was observed with a frequency of 16% in the area examined and was inversely related with loss of heterozygosity (LOH). Interestingly, most cases with MI at the region encoding for p16 were aggregated in a subgroup of breast carcinomas with no other obvious genetic and/or epigenetic *CDKN2/p16^{INK4A}* alterations. We speculate that there is an additional mechanism of *CDKN2/p16^{INK4A}* inactivation. The relationship of p16 protein level pRb, status, the p16-pRb combined immunoprofiles, and the microsatellite alterations detected at the 9p21-22 locus with the patients' clinicopathological parameters revealed two significant correlations: one between normal pRb expression and lymph node involvement ($p = 0.0263$), and the other between microsatellite alterations (LOH and or MI) and tumor size ($p = 9.2 \times 10^{-3}$).

In view of the heterogenous nature of breast cancer, we suggest that in a significant proportion of breast carcinomas, deregulation of the p16-pRb pathway in association with another, as-yet unidentified, TSG(s) of the 9p21-22 region may play a role in initiating or progressing the oncogenic procedure, while in other subgroups, alternative molecules may play this role.

Introduction

Breast carcinoma is the most common malignant tumor and the second cause of carcinoma death after lung cancer in women (1). Recently, apart from genetic factors, hormonal imbalances, and environmental parameters considered to be implicated in breast cancer development, more has been learned of the possible implications of oncogenes and oncosuppressor genes (2). It is not surprising that alterations in the molecular machinery controlling the transition from G1 to S phase might represent central events leading to breast cancer generation. In this respect, there is a strong body of evidence that cell-cycle regula-

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tors are involved in human cancer development (3).

Ongoing studies in this field revealed the p16 product of *CDKN2* gene is an important molecule involved in controlling cell-cycle progression. The *CDKN2* gene has been mapped to chromosome region 9p21 and produces two transcripts (a and b) encoding two different proteins, $p16^{INK4a}$ and $p19^{ARF}$, respectively. $P16^{INK4a}$, an 156-amino acids (aa) protein with a molecular weight of p16kDa, functions as an inhibitor of cyclin-dependent kinase 4/6 (CDKs 4/6) (4,6). Retinoblastoma protein (pRb) phosphorylation by CDKs 4/6 is a critical step in cell cycle progression. The pRb protein ($p109^{RB1}$) consists of 928aa, has a molecular weight of 105kDa, and is encoded by *RB1* tumor suppresser gene which is located on chromosome region 13q14.2 (reviewed in 7). Uncontrolled phosphorylation of pRb by CDKs 4/6 due, either to loss of functional p16 or cyclin-D1 overexpression, may lead to irregular cellular proliferation (3). The *CDKN2/INK4a* gene product seems to be a candidate tumor suppresser protein as it has been found to be non-functional in a high percentage of cell lines and various malignancies (3, 8, 9). Genetic alterations, including homozygous deletions, mutations, loss of one allele and inactivation of the other probably due to hypermethylation, appear to consist the most common mechanisms led to non-functional $p16^{INK4a}$ (2, 6, 7). A difference in the frequency of *CDKN2* genetic alterations between cell lines and primary tumors may be caused by contamination of cancerous tissue with material derived from the surrounding stromal cells and artifactual or cellular adaptation during cell culture (9–12). A similar explanation has been given for discrepancies found in pRb studies (13). In a wide spectrum of malignancies such as retinoblastomas, small cell lung carcinoma, bladder, pancreatic and breast carcinomas, pRb was inactivated by mutations (reviewed in ref. 7). Investigation of pRb and p16 protein expression at the single cell level by immunohistochemistry could be the answer to the problem of evaluating the pRb and p16 alterations using conventional molecular biology methods.

Controversy continues to surround the frequency and mechanisms of *CDKN2* gene inactivation in primary breast carcinomas (14–24). In reviewing the world literature published so far, we found four reports dealing with aberrant p16 immunoexpression and in situ distribution with percentages ranging from 37% to 73% (14–17,24). Although most studies agree that point mutations represent a rather rare mechanism of *CDKN2* inactivation (0–5%) (18,20–23,25), there is great discrepancy regarding *CDKN2* gene deletion, with figures varying from 0% to 58% (20–23,25,26). In addition, only two reports deal with the methylation status of the *CDKN2* gene in breast cancer, and they report contradicting findings (19,27). A similar discordance seems to exist concerning pRb inactivation in breast carcinomas (7–47%) (14,28–38).

It has been postulated that the p16-pRb pathway is regulated by a feedback loop involving pRb (39,40). In such a case, deregulated expression of pRb, by hyperphosphorylation, mutation, or association with viral oncoproteins, would permit high levels of p16. The existence of this inverse relationship in breast carcinomas is not clear yet (14,17,24).

The goals of the present study were to investigate the expression of p16 and pRb and their interrelationship in a series of 55 surgically resected sporadic invasive breast carcinomas; to correlate the findings with clinicopathological parameters and the hormonal status of the patients; and to look into the mechanisms of $p16^{INK4A}$ inactivation. Finally, we sought to examine the state of the chromosomal region 9p21-22 because there are indications that, in addition to *CDKN2*, $MTS2/p15^{INK4B}$, and methylthioadenosine phosphorylase (*MTAP*) tumor-suppressor genes (TSGs), it harbors another TSG(s) (26,41,42).

Materials and Methods

Tissue Samples

Specimens from 55 breast carcinomas (collected between November 1995 and December 1996) were obtained in less than 15 min after surgery. Two samples of each tumor were taken; one was snap-frozen in liquid nitrogen and stored at −70°C, the other was formalin-fixed and paraffin-embedded (FFPE). In addition, adjacent normal tissue was included from each specimen examined. The material comprised 48 ductal, 4 lobular, and 3 mixed invasive carcinomas. Microscopic grading and surgical staging of the tumors was based on the Nottingham modification of the Bloom-Richardson (43) and TNM systems (44), respectively. The age, histology, grade, tumor size, lymph node status, stage, patient's "follow-up", and estrogen (ER) and progesterone receptor (PgR) levels are presented in Table 1. None of the

tumors were from women with a strong family history of breast cancer.

Estrogen and Progesterone Receptor Status

ER and PgR levels were measured with the ligand-binding assay according to the EORTC Breast Cancer Cooperative Group procedures (45).

Immunohistochemistry

ANTIBODIES. For immunohistochemical analysis of p16 and pRb, the following antibodies (Abs) were used: C-20 (class: IgG rabbit polyclonal, epitope: residues 137–156 of the C terminus of p16) (Santa Cruz, CA); F-12 (class: IgG2a mouse monoclonal, epitope: residues 1–167 representing the full-length p16) (Santa Cruz, CA), and Rb1 (class: IgG1, mouse monoclonal, epitope: residues 375–658) (Dako, Denmark).

METHOD. First, 5-μm paraffin sections of the lesions were mounted on poly-L-lysine-coated slides, dewaxed, rehydrated, and incubated for 30 min with 0.3% hydrogen peroxide to quench the endogenous peroxidase activity. Unmasking of p16 and pRb proteins was then carried out with the heat-mediated antigen retrieval (HMAR) method, as previously described (46,47), when the MAbs, F-12 and Rb1 were applied. The immunohistochemical assay for PAb C-20 did not include antigen retrieval step. The sections were incubated with the antibodies at a 1:100 dilution at 4°C overnight. Biotin-conjugated secondary antibody was added at a 1:200 dilution for 1 hr at room temperature (RT). Next stage comprised a 20-min incubation in Strept AB Complex solution (streptavidin stock solution 1:100 and biotin-hyperoxydase stock solution 1:100 in TBS) (Dako, Denmark). For color development we used 3,3′-diaminobenzidine tetrahydrochloride (DAB) and hematoxylin as counterstain.

CONTROLS. The HeLa and Lo Vo cell lines with well-defined p16 and pRb status, respectively, were used as positive controls. Mouse IgG1 monoclonal antibody (MAb) of unrelated specificity and the IgG fraction of normal rabbit serum were used as negative controls. Furthermore, the specificity of p16 staining with the PAb C-20 was tested by incubating the latter with the control peptide C-20P, against which it was raised, and applying it on the sections. Elimination of staining verified p16 positivity.

EVALUATION. For scoring the p16 and pRb staining patterns we used previously published criteria (16,30). Cytoplasmic reactivity was disregarded and only nuclear staining above any cytoplasmic background was considered evidence of expression of the p16 and pRb proteins. The samples were divided into two categories: (*1*) p16 or pRb normal (NE), when more than 90% of the tumor nuclei were stained, and (*2*) p16 or pRb abnormal (AE), when there was absence of nuclear staining in a portion of (heterogeneous) or in the entire (homogeneous) tumor section, while admixed non-neoplastic cells showed nuclear reactivity. A mosaic pattern of staining with absence of p16 or pRb reactivity in a proportion of tumor cells was not interpreted as abnormal. If there was no discreanable nuclear staining in either tumor or stroma, the stain was regarded inconclusive.

Microdissection and DNA Extraction

DNA was extracted from adjacent 5-μm sections of each frozen tumor specimen. Contiguous 5-μm sections were processed and the first section was stained with hematoxylin and eosin to visualize the extent of the tumor cells within each sample. The boundaries of the cancerous tissue were delineated microscopically and excess normal tissues were removed with sterile surgical blades, as previously described (48). The remaining neoplastic material was digested in 500 μl lysis solution [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, containing 1% SDS, and proteinase K (Boehringer-Mannheim) at a final concentration of 100 μg/ml]. Lysis was carried out in a 55°C water bath for 48 hr. Additional proteinase K (50 μg/ml) was added on each day of lysis. DNA was extracted from the supernatant using the phenol:chloroform:isoamyl-alcohol method (49).

Microsatellite Alteration Analysis of Chromosomal Region 9p21-22

METHOD. DNA from normal and cancerous tissue was amplified by polymerase chain reaction (PCR) using a tight cluster of highly polymorphic microsatellite markers spanning 11 cM across 9p21-22 with the following order from telomeric 9p to centromeric 9p (Fig. 1A): *D9S162* (172–196 bp), *IFNA* (138–150 bp), *D9S171* (159–177 bp), and *D9S126* (226–250 bp) (Research Genetics, Hunstville, USA). The 50-μl reaction mixture contained 5 μl of DNA solution (100 ng), 10 μM Tris-HCl (pH 8.8), 50 μM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM of each dNTP

Table 1. Summary of clinicopathological features, immunohistochemical results, and 9p21 chromosomal locus LOH analysis results

Clinicopathological Features										Immuno-phenotypes		Molecular Analysis	
Sample	Age	H-P	TS	GR	ER	PR	LN	Stage	Patients' Follow-up[a]	IHC P16	IHC Rb	LOH(c)	LOH(f)
54	63	DIN	<2	II	N	N	No	I	22+	AE	AE	L	L
29	69	DIN	>2	II	P	P	No	II	28+	AE	AE	MI	MI
24	52	LIN	>2		P	P	No	II	29+	AE	AE	MI	MI
49	42	DIN	>2	II	P	P	Yes	III	22+	AE	AE	NI	H
46	67	DIN	<2	II	P	P	Yes	I	25+	AE	NE	H	H
8	80	DIN	=2	II	P	N	Yes	II	32+	AE	NE	H	H
33	64	DIN	>2	III	P	P	Yes	II	27+	AE	NE	H	H
38	67	DIN	>2	II	P	P	No	II	27+	AE	NE	H	H
40	90	Mixed	=2	II	P	N	Yes	IV	27+	AE	NE	H	H
10	61	DIN	>2	II	P	P	Yes	III	32+	AE	NE	H	MI
43	73	DIN	<2	I	UN	UN	Yes	II	26+	AE	NE	H	NI
45	45	DIN	<2	I	P	P	Yes	II	25+	AE	NE	H+MI	H
20	60	DIN	>2	II	P	P	No	II	30+	AE	NE	L	H
23	64	DIN	=2	II	P	N	No	I	29+	AE	NE	L	L
1	67	DIN	>2	III	P	N	Yes	IV	35+	AE	NE	L	L
12	39	DIN	>2	II	P	P	Yes	III	31+	AE	NE	L	L
32	66	DIN	>2	II	P	N	Yes	III	27+	AE	NE	L	L
14	78	DIN	>2	III	P	N	No	II	31+	AE	NE	L	NI
27	51	DIN	>2	II	P	P	Yes	III	28+	AE	NE	L	NI
34	70	DIN	>2	II	P	P	Yes	III	27+	AE	NE	L+MI	NI
22	57	DIN	>2	III	P	P	No	II	29+	AE	NE	MI	H
21	75	DIN	>2	II	P	P	Yes	IV	29+	AE	NE	NI	H
52	66	DIN	>2	I	UN	UN	Yes	II	22+	AE	NE	NI	H
28	55	DIN	>2	III	P	N	Yes	III	28+	AE	NE	NI	NI
26	56	LIN	<2		UN	UN	No	I	29+	AE	NE	NI	NI
47	71	Mixed	<2	II	P	P	Yes	II	24+	AE	NE	NI	NI
55	39	Mixed	<2	II	N	P	Yes	II	22+	NI	AE	NI	NI
53		LIN	>2		P	P	Yes	IV	22+	NI	NE	L	NI
13	57	DIN	<2	I	P	P	No	I	31+	NI	AE	NI	H
25	74	DIN	>2	III	P	N	Yes	II	29+	NI	NE	L	NI
9	38	DIN	<2	II	P	P	Yes	I	32+	NE	AE	H	H
2	81	DIN	=2	II	P	P	No	I	33+	NE	AE	H	H
6	69	DIN	=2	II	P	P	Yes	II	32+	NE	AE	H	H
30	77	DIN	>2	III	N	N	No	II	28+	NE	AE	H	H
39	88	DIN	>2	II	P	P	No	II	27+	NE	AE	H	H
42	54	DIN	>2	II	P	P	No	II	26+	NE	AE	H	H
11	81	DIN	<2	I	P	P	No	I	32+	NE	AE	H	NI
18	65	DIN	=2	III	P	P	Yes	III	30+	NE	AE	H	NI
31	73	DIN	=2	II	P	N	Yes	II	28+	NE	AE	NI	H
16	53	DIN	<2	III	P	N	Yes	III	31+	NE	AE	NI	NI
3	58	DIN	>2	III	N	N	No	II	32+	NE	NI	NI	NI
41	64	DIN	<2	II	UN	UN	Yes	II	26+	NE	NE	H	H
44	66	DIN	<2	I	N	N	No	I	25+	NE	NE	H	H
15	48	DIN	=2	I	P	N	Yes	II	30+	NE	NE	H	H
35	72	DIN	=2	I	P	P	Yes	I	27+	NE	NE	H	H
7	46	DIN	>2	II	P	P	Yes	I	32+	NE	NE	H	H
36	62	DIN	>2	II	P	P	Yes	III	27+	NE	NE	H	H
37	65	DIN	>2	III	P	N	Yes	III	27+	NE	NE	H	H

Table 1. Summary of clinicopathological features, immunohistochemical results, and 9p21 chromosomal locus LOH analysis results *(cont.)*

	Clinicopathological Features									Immuno-phenotypes		Molecular Analysis	
Sample	Age	H-P	TS	GR	ER	PR	LN	Stage	Patients' Follow-up[a]	IHC P16	IHC Rb	LOH(c)	LOH(f)
4	66	DIN	>2	II	N	N	Yes	III	32+	NE	NE	H	L
17	66	DIN	>2	III	P	P	Yes	III	30+	NE	NE	H	MI
5	60	DIN	<2	II	P	P	No	I	32+	NE	NE	H	NI
19	64	LIN	>2		P	P	Yes	III	30+	NE	NE	H+MI	H
51	60	DIN	=2	I	P	N	Yes	I	22+	NE	NE	L	H
48	75	DIN	=2	II	UN	UN	Yes	II	24+	NE	NE	NI	H
50	35	DIN	=2	III	N	P	Yes	II	22+	NE	NE	NI	H

Abbreviations: H-P, histology; TS, tumor size (in cm); GR, grade; ER/PR, estrogen and progesterone receptor estimation, respectively; LN, lymph nodes metastases; IHC, immunohistochemistry; DIN, ductal invasive carcinoma; LIN, lobular invasive carcinoma; N, negative (normal expression); P, positive (overexpression); UN, undetected level; AE, aberrant expression; NI, noninformative; NE, normal expression; L, LOH loss of heterozygosity; MI, microsatellite instability; H, heterozygous.

[a]Note: The number indicates months of survival after surgery, (+) indicates that the patient is alive.

(dATP, dCTP, dGTP, and dTTP), 1 μM of each marker pair, and 1.5 units of *Taq* DNA polymerase (Promega, Madison, USA). The thermal cycle profile was denaturation at 95°C for 10 min before the addition of *Taq* polymerase, followed by 30 cycles with incubations of 40 sec at 95°C, 35 sec at 56°C, and 30 sec at 72°C. The products were analyzed on 10% polyacrylamide gels containing 10% glycerol. Visualization was carried out by silver staining (49).

CONTROL. To determine whether the PCR product was a microsatellite region, denaturating polyacrylamide gels were used. The bands corresponding to the microsatellite regions, due to the higher existence of dinucleotide repeats, are separated according to their molecular weight. Furthermore, all samples showing changes in microsatellite sequences were verified by subjecting the corresponding DNA sample to a second, independent PCR analysis. This, as well as control PCR reactions lacking DNA, were done to eliminate the chance of false-positives due to PCR artifacts or sample contamination. Finally, for microsatellite instability (MI) determination, we used previously published criteria (50).

Methylation Analysis of the CDKN2 Gene

PRIMERS. A 239-bp fragment of exons 1a was amplified using the following primers, employing the Oligo Software v.4.01: p16AU: 5'-GGAGAGGGGGAGAGCAGGCA-3', p16AD: 5'-CTCCAGAGTCGCCCGCCATC-3'.

METHOD. The inability of some restriction enzymes to cut methylated sequences was used to examine the methylation status of exon 1a. There are two *Hpa*II sites and one *Ksp*I site (both restriction enzymes are methylation-sensitive) within exon 1a. One microgram of genomic DNA from matched normal and tumor samples was digested overnight with *Hpa*II and *Ksp*I as previously described (51). Five microliters of the digested DNA solutions was used as a template in a 50-μl reaction mixture containing 10 μM Tris-HCl (pH 8.8), 50 μM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 5% DMSO, 5% glycerol, 200 μM of each dNTP (dATP, dCTP, dGTP and dTTP), 1 μM of each primer, and 1.5 units of *Taq* DNA polymerase (Promega). The thermal cycle profile was denaturation at 95°C for 10 min before the addition of *Taq* polymerase, followed by 30 cycles with incubations of 1 min at 95°C, 45 sec at 57°C, and 45 sec at 72°C. PCR products were separated on 2% agarose gels.

EVALUATION OF CONTROLS. When exon 1a of the *CDKN2* gene is methylated, the methylation-sensitive restriction enzyme fails to cut and a PCR product is obtained. For each specimen analyzed, undigested and non-methyl-sensitive *Msp*I-digested samples served as positive and negative controls, respectively, as previously described (51). Finally, as methylation-positive samples we evaluated only the cases with reproducible results.

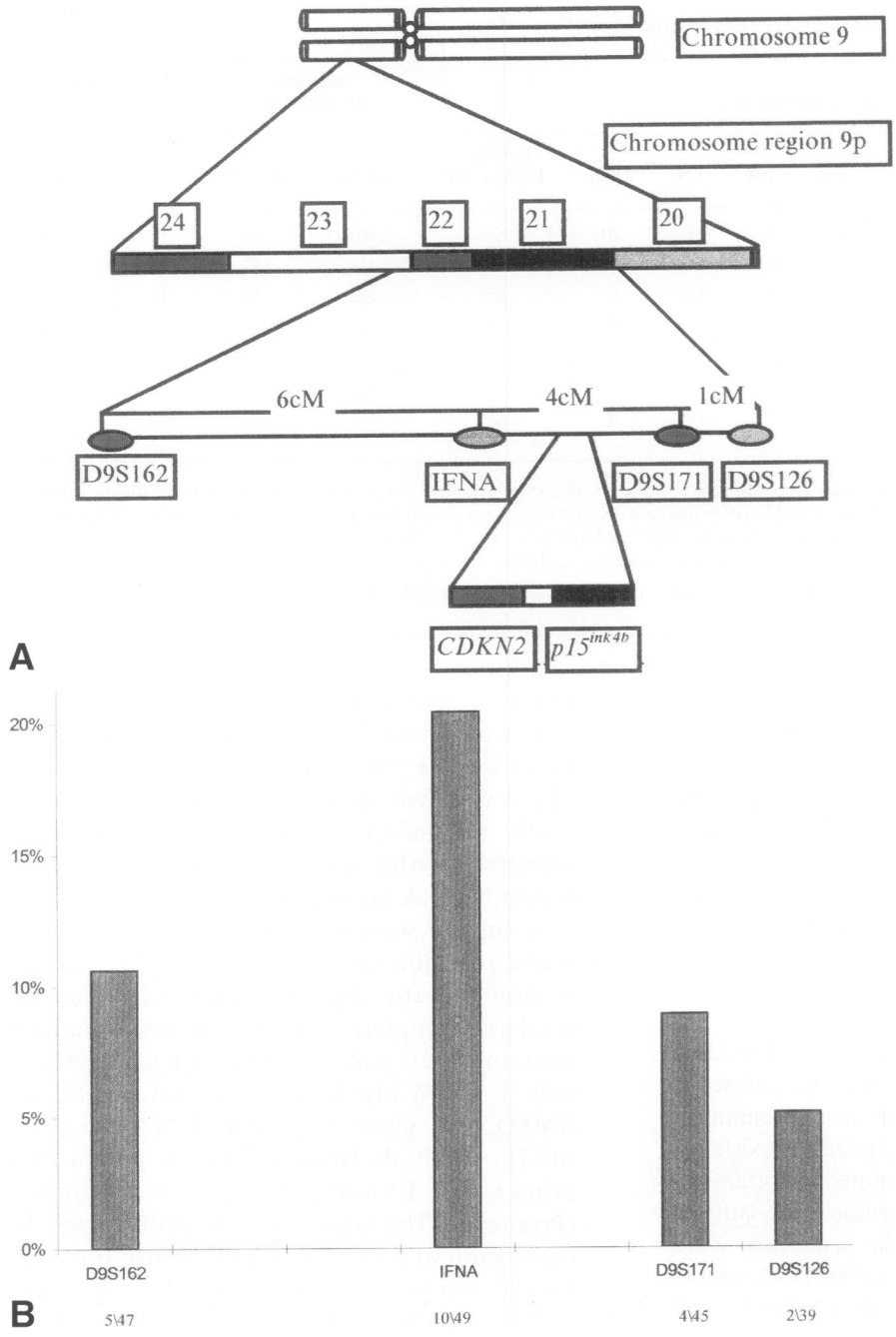

Fig. 1. (A) Schematic representation of the 9p21-22 region, the polymorphic microsatellite markers used in the study, and the location of the *CDKN2* and *p15^{ink4b}* genes. (B) Deletion frequencies observed at the *D9S162, IFNA, D9S171,* and *D9S126* loci.

Single-Strand Conformation Polymorphism Analysis of CDKN2/p16^{INK4A} *Gene*

PRIMERS. The primers used for methylation analysis of exon 1a: p16BU: 5′-GGCTCTGACCATTCTGTTCTCTC-3′. p16BD: 5′-GGCTGAACTTTCTGTGCTGG-3′, designed by using Oligo Software v.4.01, for exon 2 of *CDKN2/p16^{INK4A}* (419 bp PCR-amplified fragment) were used for mutation analysis.

METHOD. Single-strand conformation analysis was performed on normal and tumor tissue–derived DNA as previously described (52).

Statistical Analysis

Statistical analysis was based on the chi(χ^2)-test with Yates' correction. An additional two-tailed Fisher's exact test was applied only when the number of samples in any cell of a given statis-

tical table was ≤5. The statistical difference was considered significant if $p < 0.05$.

Results

Immunohistochemistry

IMMUNOHISTOCHEMICAL EXPRESSION OF p16 AND pRb PROTEINS AND RELATIONSHIP WITH VARIOUS CLINICOPATHOLOGICAL PARAMETERS. Immunohistochemical analysis revealed aberrant expression of p16 and pRb in 26 (47%) and 16 (29%) of the 55 carcinomas, respectively (Table 1; Figs. 2, 3). There was concordance of p16 staining with both antibodies used. In four cases, staining was regarded inconclusive. P16 and pRb results and their relation to age, grade, tumor size, lymph node status, stage, and hormonal status are presented in Table 2. Statistical analysis did not reveal any significant correlations with the exception of an association between pRb expression and lymph node status [pRb(No)/LN(+) 30/37 (81%) vs. pRb(No)/LN(−) 8/17 (47%) $p = 0.0263$].

INTERRELATION BETWEEN p16 AND pRb EXPRESSION. There was a statistical trend pointing out an inverse relation between p16 and pRb expression. Breast carcinomas with strong pRb expression showed frequently abnormal p16 staining (22/50), wheras tumors with aberrant pRb expression were more likely to be positive for p16 (10/50) [pRb(No)/p16(Ab): 14/24 (58.3%) vs. pRb(No)/p16(No): 22/26 (84.6%) $p = 0.079$].

RELATION BETWEEN COMBINED p16-pRb IMMUNOPROFILES AND VARIOUS CLINICOPATHOLOGICAL PARAMETERS. The statistical analysis did not show any significant associations as presented in Table 2.

Microsatellite Alteration Analysis of Chromosomal Region 9p21-22

For microsatellite analysis (MA) analysis we used a tight cluster of highly polymorphic microsatellite markers spanning 11 cM across 9p21-22 with the following order from telomeric 9p to centromeric 9p (Fig. 1A): *D9S162, IFNA, D9S171,* and *D9S126* (Fig. 1A).

Out of 55 cases, 47 (85%) were informative for *D9S162,* 49 (89%) for *IFNA,* 45 (82%) for *D9S171,* and 39 (71%) for *D9S126.* Thirteen (24%) breast carcinomas displayed loss of heterozygosity (LOH) in one or more markers (Fig. 4A). The deletion frequencies for each marker are presented in Figure 1B. The results were further categorized into three groups. Group LOH(c), the cases demonstrating a central LOH at the *IFNA* and/or *D9S171* loci, indicating a possible deletion of the *CDKN2* gene; group LOH(f), the patients with chromosomal abnormalities at the flanks upstream of *IFNA* and/or downstream of *D9S171;* and group LOH(−), the cases with no indication of LOH and/or noninformative (NI) samples. Seven of the 55 patients were placed in group LOH(c), 1 in group LOH(f), 5 in both groups: LOH(c) and LOH(f), and 42 in group LOH(−) (Table 1).

Apart from chromosomal deletions, 9 (16%) patients demonstrated MAs, which appeared as an expansion or a compression of a single or

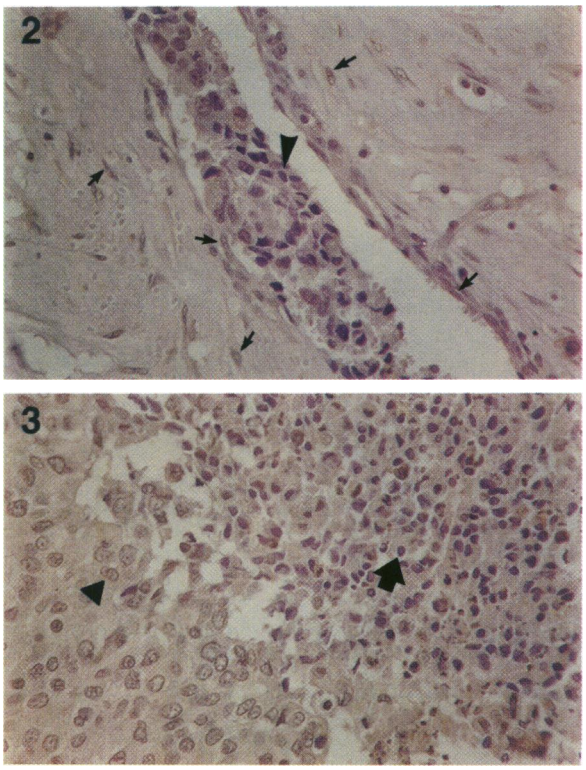

Fig. 2. Ductal breast carcinoma with homogenous aberrant p16 expression (tumor region indicated by arrowhead). Stromal cells (fibroblasts) with normal p16 staining (arrows). Streptavidin-biotin peroxidase technique with F-12 anti-p16 antibody (see Materials and Methods) and hematoxylin counterstain (×500).

Fig. 3. Ductal breast carcinoma with heterogenous aberrant pRb expression (the area with pRb normal expression is indicated with an arrowhead whereas the absence of pRb expression is indicated by an arrow). Streptavidin-biotin peroxidase technique with Rb1 anti-pRb antibody (see Materials and Methods) and hematoxylin counterstain (×500).

Table 2. Relationship of immunohistochemical results with various clinicopathological parameters

	Tumor Size (cm)				Lymph Node Metastasis			Grade				Stage					ER			PR		
IHC	<2	2	>2	*p*	No	Yes	*p*	I	II	III	*p*	I	II	III	IV	*p*	P	N	*p*	P	N	*p*
p16																						
NE	6	9	10		8	17		5	13	7		7	10	8	0		7	18		11	14	
AE	6	3	17	0.091	9	17	0.921	3	18	5	0.445	4	12	7	3	0.256	4	22	0.45	11	15	0.87
Total	12	12	27		17	34		8	31	12		11	22	15	3		11	40		22	29	
pRb																						
NE	8	8	22		8	30		7	22	9		7	16	11	4		8	30		18	20	
AE	6	4	6	0.340	9	7	0.026	2	11	3	0.749	5	7	4	0	0.460	3	13	0.85	4	12	0.22
Total	14	12	28		17	37		9	33	12		12	23	15	4		11	43		22	32	
p16/pRb																						
NE/NE	3	5	6		2	12		4	7	3		4	5	5	0		5	9		7	7	
NE/AE	3	4	3		5	5		1	6	3		3	4	3	0		1	9		3	7	
AE/NE	5	3	14	0.406	6	16	0.068	3	14	5	0.573	3	10	6	3	0.779	3	19	0.33	10	12	0.67
AE/AE	1	0	3		3	1		0	4	0		1	2	1	0		1	3		1	3	
Total	12	12	26		16	34		8	31	11		11	21	15	3		10	40		21	29	

Abbreviations: IHC, immunohistochemistry; NE, normal expression; AE, aberrant expression; ER/PR, estimation of estrogen and progesterone receptor expression, respectively; P, positive (overexpression); N, negative (normal expression).

ladder of bands (Fig. 4B). These alterations were characterized as microsatellite instability (MI) as previously described (50,53) MI was observed in all the microsatellite loci examined. In one of these cases, MI coexisted with LOH. Thus, in total, MAs (LOH and/or MI) at the 9p21-22 chromosomal region were detected in 20 (36%) cases.

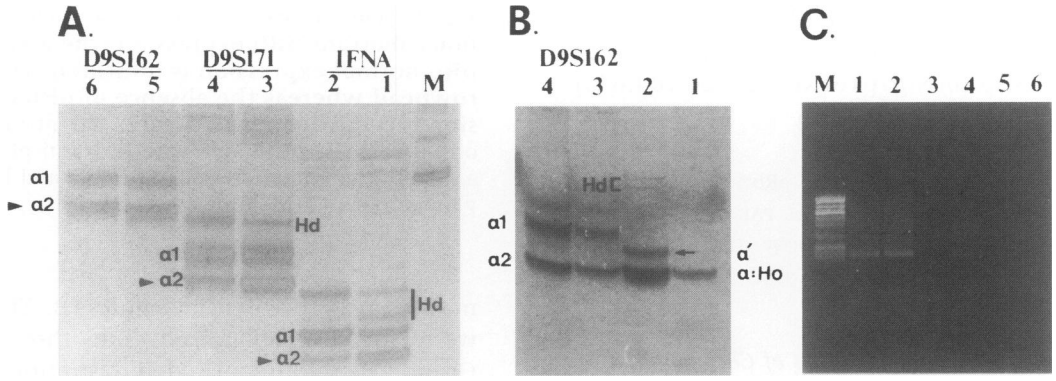

Fig. 4. Representative results of deletion mapping and microsatellite instability analysis with chromosome markers *D9S162, D9S171,* and *IFNA* and representative results of the PCR-based methylation assay. (A) M, marker (PUC19/*Sau*3AI). Lanes 1–6, matched normal tumor samples (case 12) with polymorphism at the *D9S162, D9S171,* and *IFNA* loci and indication of loss of heterozygosity (LOH); (arrowhead). a1 and a2, alleles; Hd, heteroduplex lanes; LOH is shown with arrowhead). (B) Lanes 1 and 2, matched normal tumor samples (case 17) with no polymorphism at the *D9S162* locus (Ho, homozygous), although the tumor sample showed MI, which is demonstrated as an aberrant band (α'); (arrow). Lanes 3 and 4, matched normal tumor samples with heterozygosity at *D9S162* locus (case 33). α1 and α2, alleles; Hd, heteroduplex lanes. (C) De novo methylation of *CDKN2/p16*^{INK4a} exon 1a in a ductal breast carcinoma (case 8). M, marker (PUC19/*Sau*3AI). Lanes 1 and 2, undigested PCR products from matched normal tumor sample. Lanes 3 and 4, digestion with methylation-sensitive enzyme *Hpa*II revealed that *CDKN2/p16*^{INK4A} in non-tumor tissue is unmethylated as no amplification by PCR is seen, whereas in the tumor, *CDKN2/p16*^{INK4A} is methylated as digestion does not affect amplification. Lanes 5 and 6, digestion with non-methylation-sensitive enzyme *Msp*I affects amplification in normal and tumor tissue.

In relation to clinicopathological parameters, we observed a highly significant association between MAs and tumor size ($p = 9.2 \times 10^{-3}$) and a probably significant correlation between MI and tumor size ($p = 0.0526$) (Table 3).

CDKN2/p16^{INK4A} Gene Methylation Analysis

To address further the molecular basis of aberrant p16 expression, we examined the methylation status of *CDKN2/p16^{INK4A}* exon 1a, which has been shown to correlate strongly with transcriptional silencing of the gene (54). We found that 11 (20%) of the 55 carcinomas analyzed exhibited de novo methylation of exon 1a (Fig. 4C). Methylation patterns did not vary between *Hpa*II and *Ksp*I. Furthermore, all normal tissues were unmethylated within the examined regions.

SSCP Analysis of CDKN2/p16^{INK4A} Gene

To assess point mutations and short nucleotide sequence insertions or deletions of the *CDKN2/p16^{INK4A}* gene, we used SSCP to analyze exons 1a and 2, obtained by PCR, from tumor and normal tissue–derived DNA. No tumor-specific migrational shifts were observed in any of the breast carcinomas studied.

Relation between p16 Immunohistochemical Findings, Microsatellite Alterations at 9p21-22 Locus, and Methylation Status of CDKN2/p16^{INK4A} Gene (Fig. 5)

The cases with abnormal p16 (AE) expression were placed in group A and the cases with normal p16 staining (NE) in group B. Group A was further divided into four subgroups according to the LOH(c) and methylation analysis results and group B into three subgroups according to LOH status. The carcinomas with inconclusive immunostaining were placed in group C.

Statistical analysis revealed a correlation between aberrant expression of p16 protein, MAs, and LOH at the 9p21-22 locus, respectively [MAs/p16(AE) 14/26 (54%) versus MAs/p16(NE) 4/25 (16%), $p = 0.01$ and LOH/p16(AE) 9/26 (35%) versus LOH/p16(NE) 2/25 (8%), $p = 0.04$]. This association was even higher in the patients in group LOH(c) [LOH(c)/p16(AE) 9/26 (35%) versus LOH(c)/p16(NE) 1/25 (4%), $p = 0.01$]. All the cases with de novo *CDKN2/p16^{INK4A}* methylation were accompanied with abnormal p16 staining (subgroups A2 and A3). Although the samples comprising subgroup A4 did not show either LOH(c) or abnormal methylation status or SSCP tumor-specific shifts of *CDKN2/p16^{INK4A}*, four of them demonstrated microsatellite instability.

Discussion

To the best of our knowledge, the results of our study, dealing mainly with the relation between abnormalities of the p16-pRb pathway and alterations at the 9p21-22 region in sporadic invasive breast carcinomas, have not been reported so far.

The p16-pRb pathway was investigated by immunohistochemistry, which allows evaluation of protein expression at the single-cell level (16,17,30). In our approach, a mosaic pattern of staining was not interpreted as abnormal since, even in p16- or pRb-positive cell lines, a subset of nuclei remained unstained because of cell-cycle fluctuations (55,56). This can be explained by the findings that levels of p16 may vary as much as 5-fold during a cell cycle, the lowest occuring in G0 and early G1 phase (55). A similar situation has been described in pRb levels (56). In our study, sole cytoplasmic reactivity of p16 was disregarded. Its significance is unclear and has been observed by others (17). Interestingly, in a recent study, Marsh and Varley reported exclusive cytoplasmic p16 expression in 21 out of 41 (51%) breast lesions (24). Nevertheless, if cytoplasmic reactivity proves to be specific, one possible explanation for this could be that alteration in subcellular localization could represent an additional mechanism of p16 inactivation. Wild-type p53 protein has an analogous mechanism of loss of function (57).

Aberrant p16 was observed in 47% of the carcinomas. This is consistent with the findings of Gerardts and Wilson, who observed abnormal p16 protein staining in 49% of the breast carcinomas studied (16), while others have reported percentages ranging from 37% to 73% (14,15,17,24). This discrepancy may be due to technical parameters of the assays and/or criteria of positivity. Furthermore, assessment of p16, as well as pRb, in breast tissue is challenging because the latter is hard to cut and process in pathology laboratories, thus leading frequently to prolonged fixation, which is responsible for "antigen loss." P16 protein appears to be particularly susceptible to fixation solutions and this may explain the inconclusive staining results observed by us and others (16).

To investigate the mechanisms that underlie *p16^{INK4A}* inactivation, we examined the chromo-

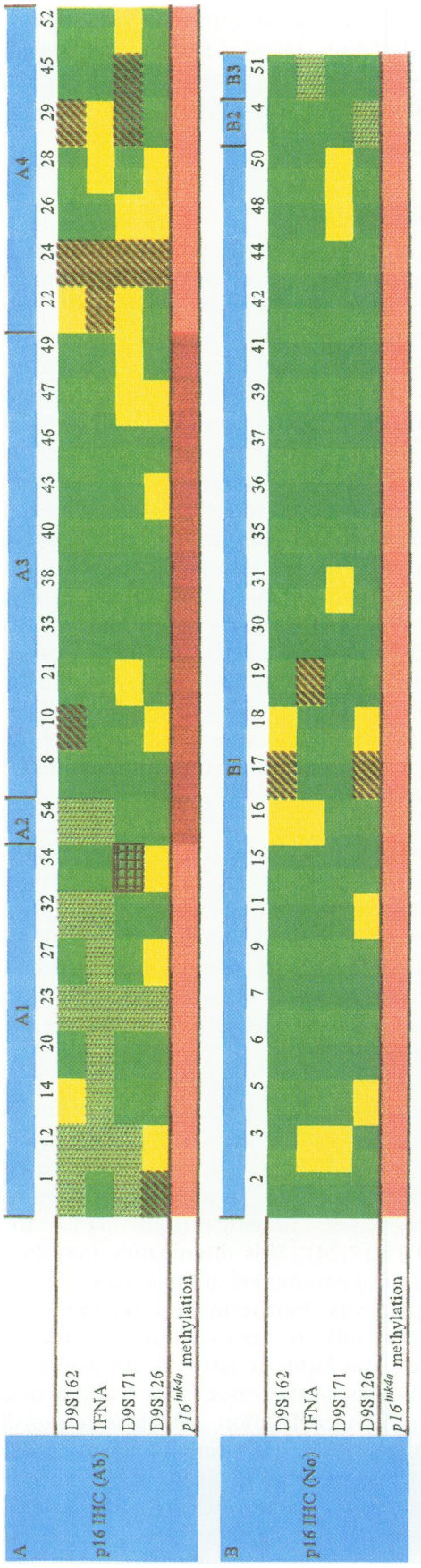

Fig. 5. Relation between p16 immunohistochemistry, microsatellite alterations at the 9p21-22 chromosome region, and methylation status of the *CDKN2/p16*^{INK4A} gene.

Table 3. Relationship of alterations at 9p21-22 chromosomal region with various clinicopathological parameters

	Tumor Size (cm)				Lymph Node Metastasis			Grade				Stage					ER			PR		
IHC	<2	2	>2	*p*	Yes	No	*p*	I	II	III	*p*	I	II	III	IV	*p*	P	N	*p*	P	N	*p*
MA																						
+	2	2	16		13	7		2	10	5		3	7	7	2		2	14		7	9	
−	12	10	13	0.0091	24	11	0.7860	7	19	8	0.7149	10	17	7	2	0.3971	5	29	0.8203	11	23	0.6402
Total	14	12	29		37	18		9	29	13		13	24	14	4		7	43		18	32	
LOH																						
+	1	2	10		9	4		1	8	3		3	3	5	2		2	8		3	7	
−	13	10	19	0.1151	28	14	0.8633	8	21	10	0.9202	10	21	9	2	0.2286	5	35	0.9188	15	25	0.9413
Total	14	12	29		37	28		9	29	13		13	24	14	4		7	43		18	32	
MI																						
+	1	0	8		6	3		1	3	3		0	4	4	1		0	8		4	4	
−	13	12	21	0.0527	31	15	0.9662	8	26	10	0.5242	13	20	10	3	0.2320	7	35	0.4906	14	28	0.6183
Total	14	12	29		37	18		9	29	13		13	24	14	4		7	43		18	32	

Abbreviations: MA, microsatellite alteration; LOH, loss of heterozygosity; MI, microsatellite instability; +/−, presence or absence of specified genetic alteration; ER/PR, estimation of estrogen and progesterone receptor expression, respectively; P, positive (overexpression); N, negative (normal expression).

somal region 9p21-22 by LOH analysis using closely spaced microsatellite markers (Fig. 1A) and the methylation and structural status of the $p16^{INK4A}$ gene by a PCR-based methylation assay and PCR-SSCP analysis, respectively. The findings were then correlated with the results of immunohistochemistry. A statistically significant association was observed between LOH(c) and abnormal p16 protein staining ($p = 0.01$) (Fig. 5), suggesting that deletions may represent an important mode of $p16^{INK4A}$ gene inactivation. Several groups have investigated $p16^{INK4A}$ gene deletions in breast tumors, using Southern blotting and dublex-PCR assays, producing controversial results (20–23,25). These differences may be due to contamination of the homogenates by non-neoplastic cells and aneuploidy of tumor cells. The current microdissection techniques have ameliorated but not completely solved this problem. Furthermore, we observed that 42% of the breast carcinomas with aberrant p16 expression were hypermethylated, supporting the hypothesis that methylation of the 5'CpG island within the first exon of the $p16^{INK4A}$ gene correlates with transcriptional silencing (subgroups A2 and A3, Fig. 5) (54). In addition, no methylation of $p16^{INK4A}$ in adjacent normal breast tissue was observed. Our findings are in accordance with the results of Herman et al., although, they did not mention evaluation of $p16^{INK4A}$ methylation in normal breast tissue (19). In contrast, Van Zee and co-workers demonstrated that normal breast tissue exhibits $p16^{INK4A}$ methylation more frequently than breast carcinoma, suggesting that its expression in carcinoma cells may represent an unsuccessful attempt to limit growth in cells lacking some other normal mechanisms of control (27). Similar discordances are reported in studies concerning the methylation status of $p16^{INK4A}$ in colon cancer tissue and normal colonic mucosa (27,58,59). Contamination of the normal epithelial cells with cells of different origin or partial digestion with the methyl-sensitive enzymes used could account for the aforementioned differences. PCR-SSCP analysis of exons 1a and 2 in our study did not reveal any minor structural alterations, which supports previous evidence suggesting that intragenic $p16^{INK4A}$ alterations are infrequent events in breast carcinomas (20–23,25). Thus, overall, 19 of the 26 cases (73%) with abnormal p16 protein staining had LOH(c) and/or abnormal methylation status of the *CDKN2*/$p16^{INK4A}$ gene (Fig. 5). Therefore, taking together our data and those reported thus far, it appears that deletions and transcriptional silencing by methylation might be the predominant mechanisms that inactivate $p16^{INK4A}$ gene in breast carcinomas. The seven cases (27%) of subgroup A4 with loss of p16 did not show obvious genetic and epigenetic alterations (Fig. 5). One possible explanation for aberrant p16 expression in these cases is that the

microsattelite markers used were not sufficient to detect small deletions in the *CDKN2/p16*^{INK4A} region. Alternatively, the presence of normal contaminating DNA may contribute to a retention pattern in cases with homozygous deletion. A third possibility that could account for loss of protein expression is mutations outside the examined region; such is the case of the mutation being in the second intron splice donor site, which results in a smaller p16 protein with a reduced half-life, possibly undetectable by immunohistochemistry (60). The retention of p16 protein expression in case 51 (subgroup B3) suggests either alterations of another TSG(s) located in the 9p21-22 region and/or a compensatory mechanism by the remaining *CDKN2/p16*^{INK4A} allele.

Deletion mapping studies have shown that the frequency of alterations of the 9p21-22 region in primary breast tumors are higher than those reported for *CDKN2/p16*^{INK4A} (20–23, 25,26). In the present study LOH was observed in 24% of the samples, which is lower with the findings presented by the other groups (22,26) and comparable to the percentage of LOH(c) (22%). Despite the low frequency of losses flanking the *CDKN2/p16*^{INK4A} area (11% at *D9S162* and 5% at *D9S126*), 83% of them were accompanied by LOH(c). The *D9S162* and *D9S126* loci are placed 6 cM and 1 cM away from *IFNA* and *D9S171*, respectively, and have been proposed to lie near the location of putative TSG(s) (41,60,61). The *talin* gene has recently been mapped in this region (62) and its product is a critical molecule for the formation of focal adhesions. Inactivation of *talin* disassembles many of these structures (63). *Talin*, therefore, is a gene candidate for a role in breast carcinogenesis. It is possible that co-deletion of this candidate TSG(s) with *CDKN2/p16*^{INK4A} is important in the development of a subset of invasive breast carcinomas. Marsh and Varley noticed that LOH at *D9S162* was more commonly observed in ductal in situ carcinomas (DCIS) and invasive carcinomas with a DCIS component (41%) than solely invasive carcinomas (10%) (26). Moreover, in a recent study we found a high deletion frequency at loci *D9S162* and *D9S126* in non-small-cell lung carcinomas (64). Taking together our data and those reported thus far, we can speculate that if an additional TSG(s) exists in the 9p21-22 region, its involvement in carcinogenesis is probably tissue- and histology-dependent. In addition to LOH, microsatellite analysis revealed microsatellite instability (MI) in 16% of carcinomas. Only one tumor exhibiting MI showed evidence of LOH, a finding in accordance with the inverse correlation found between MI and LOH in colorectal cancer (65). MI provides a marker for mutator or replication error phenotype (RER+), a recently defined manifestation of genetic instability. It has been observed in both hereditary and sporadic cancers, and it is caused by a deficiency of DNA mismatch repair genes (MMR) (66). The inability to detect DNA mismatches during replication can result in structural gene alterations that can lead to deregulated function of the cell-cycle control machinery. For example, mutations in the transforming growth factor β (TGF-β) type II receptor have been found in colon cancer cell lines with MI, resulting in enhanced tumorigenicity (67). In a similar manner, the high frequency of MI observed in subgroup A4 may represent an additional mechanism of *CDKN2/p16*^{INK4A} gene deregulation. The data concerning MI in breast cancer are conflicting with respect to the frequency and affected repeat sequences (68–73). Considering the frequency, our percentage is closer to that of Wooster et al. (69) and Yee et al. (70), who found MI in 10% and 20% of the cases examined, respectively. In two other reports, MI occured in 5% (68) and 30% (71) of breast carcinomas. High levels of MI were also noted by Patel et al. for specific loci on chromosomes 2p, 8p and 10p (72), while Glebov et al. noticed differences in MI between tumors with a family history and sporadic breast cancers (73). As far as the repeat sequence pattern is concerned, we observed that dinucleotide repeats are frequently affected, although we did not include tri- and tetranucleotide motifs in our study. This finding is in agreement with the results of Yee et al. (70) but differs from those of others where MI was noted mainly in trinucleotide (68,69) and tetranucleotide repeats (71). More studies are needed to clarify whether these variations in susceptibility of microsatellite sequences reflect the involvement of different mutated MMR genes, different mechanisms for generating MI, or simply differences in experimental design.

Abnormal loss of pRb expression was found in 29% of our cases, which is in accordance with the reported incidence of other series (14,32–35,37). Serrano et al. have proposed a negative feedback model, showing that inactivation of pRb during G1 leads to increased p16 expression to limit CDK4 activity (5). Several studies in various tumors, including breast, have demonstrated a reciprocal relation between pRb and p16 (74–77). In this cohort we noticed a statistical trend pointing out the inverse correlation between p16 and pRb expression (p = 0.079)

which would agree with the proposed model. However, 29% of the cases with reduced expression of pRb showed corresponding aberrant p16 staining. Recently, Marsh and Varley (24) also reported simultaneous abnormal p16 and pRb expression in a significant proportion (41%) of breast lesions. A similar phenomenon has been noted by Wang and Becker in the melanocytic system (78), by Hangaishi et al. in primary lymphoid malignancies (79), and by our group in non-small-cell lung carcinomas (64). Since several lines of evidence suggest that functional pRb is essential for cell-cycle inhibition by p16 (80), inactivation of p16 in a cell without functional pRb is not likely to confer additional growth advantage to the cancerous cell. In carcinomas with double hits, one possible explanation is that p16 inactivation is an early event followed by subsequent loss of pRb. This mechanism could contribute to advantageous tumor growth by cancelling all the incoming inhibitory signals to pRb from the other cyclin-dependent kinase inhibitors (CDKIs) (81). In this respect, our findings that p16 inactivation was noticed in almost half of the carcinomas and its frequency was higher than loss of pRb, in addition to the lack of association with tumor stage, suggest an early involvement of *CDKN2* alterations in breast cancer development. This can be further supported by the study of Dublin et al., who also did not notice an association between loss of p16 and clinical stage in breast carcinomas (14). Alternatively, p16 may participate in other unknown pRb-independent pathways. This assumption can be sustained by the finding that p16 inactivation causes stimulation of cyclin D2 and D3, which may act on other substrates besides pRb (81).

The relation between p16 status, pRb protein level, the p16-pRb immunophenotypes, and the alterations detected at the 9p21-22 locus with the patients' clinicopathological parameters revealed only two significant correlations: one between positive pRb reaction and lymph node involvement ($p = 0.026$), and the other between microsatellite alterations and tumor size ($p = 9.2 \times 10^{-3}$). Our observation regarding strong pRb expression and lymph node metastases is surprisingly unusual. One would expect loss (rather than increased expression) of an oncosuppressor to be associated with an adverse prognostic parameter. A number of studies in breast cancer have examined the association of pRb with various prognostic parameters and have reported controversial findings. In a large series of breast carcinomas, Trudel et al. found an association between strong pRb reaction and high nuclear grade tumors (38), while Berns et al. observed that *RB1* gene alterations were significantly more frequent in node-negative patients and T1 tumors (34). No relation among various clinical parameters, including grade, nodal status, stage or receptor status, was found by Thorlacius et al. (82). In contrast, the results of other works have shown a correlation between *RB1* alterations and/or protein loss and poor prognostic indicators, such as poorly differentiated tumors (14,37), positive lymph node status (36), and increased proliferative activity (35). With respect to the relation between *RB1* alterations and survival in breast cancer, the few published studies have shown no association. From these contrasting data, it is difficult to draw a specific picture of the role of the *RB1* gene in sporadic breast cancer. The conflicting results could be due to the variety of techniques used to estimate *RB1* status and expression. The accuracy of molecular studies may be affected by normal tissue "contamination", while immunohistochemistry has the advantage of evaluating protein expression at the single-cell level. However, in the case of pRb, immunohistochemistry gives no indication of the protein's phosphorylation status and therefore, of its activity state, nor does it distinguish mutant from wild-type pRb proteins. Hence, it is possible that this could be the case in certain samples of our study. With regard to our second significant finding, there has been no previous report of a correlation between microsatellite alterations at the 9p21-22 chromosomal region and a poor prognostic indicator, such as tumor size. Interestingly, we also observed a probably significant association between MI and tumor size ($p = 0.05$), which is in keeping with the work of Paulson et al., who demonstrated a correlation with factors commonly related to poor prognosis (71). But this finding contrasts with other studies suggesting that MI is an early event in mammary tumorigenesis (68,70). Finally, the data concerning the relation between p16 and prognostic features are few (14,15). Dublin et al. unexpectedly found a strong association between p16-positive staining and poor prognosis (14), while Ito et al. reported that abnormal p16 immunoreactivity was more frequent in tumors of larger size and/or higher stage (15).

In view of the heterogenous nature of breast cancer, the large number of genetic targets recognized, and the overlapping and compensatory pathways linking most of them, we suggest that in a significant proportion of breast carcinomas,

deregulation of the p16-pRb pathway in association with other unidentified TSG(s) of the 9p21-22 region may play a role in initiating or advancing the oncogenic procedure, whereas in other subgroups, alternative molecules may play this role.

Acknowledgments

This work was funded by National Greek Health Committee (KESY) grant Y3α/2442/12-11-97 for research protocols and "Lilly" pharmaceutical company.

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