Altered *WAF1* Genes Do Not Play a Role in Abnormal Cell Cycle Regulation in Breast Cancers Lacking *p53* Mutations[1]

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ABSTRACT

Seventy-five to 80% of breast cancers are negative for *p53* gene mutations. We have investigated the possibility that altered *WAF1* genes provide an alternative mode of cell cycle disruption in these tumors. DNA from a total of 85 primary breast tumors and cell lines from both the United States and Australia were examined for *WAF1* and *p53* mutations. With the exception of one primary tumor containing the polymorphic codon 31 (AGC→AGA), no missense mutations in the *WAF1* gene were found in 33 primary tumors or in the 19 cell lines from the United States. By contrast, 2 of 33 tumors from Australia contained tumor-specific missense mutations in the *WAF1* gene, while an additional six cases contained the AGC→AGA polymorphic 31st codon in the *WAF1* gene. The *p53* mutation frequency in the Australian cohort (18%) was found to be similar to that reported by us (Glebov *et al.*, Cancer Res., *54:* 3703–3709, 1994; Runnebaum *et al.*, Proc. Natl. Acad. Sci. USA, *88:* 10657–10661, 1991) in the tumors of United States patients (13%) with sporadic breast cancer. Thus, mutations in the *WAF1* gene are rare in tumors with or without *p53* mutations, suggesting that except in a minor population of breast cancer patients of Caucasian origin, cell cycle dysregulation by mutated *p53* or *WAF1* genes may not contribute to breast tumor initiation or progression.

INTRODUCTION

The *WAF1/CIP1* gene encodes p21, a cell cycle-dependent kinase inhibitor that binds to and inhibits multiple cyclins, including cyclin D/Cdk4, cyclin E/Cdk2, and PCNA;[3] these interactions serve to block the entrance of cells from G_1 to S (1, 2). *WAF1* is transcriptionally regulated by *p53*, a tumor suppressor gene that has been identified as a target for mutation in a wide variety of tumors including breast cancer (3). In the cell, *p53* is rapidly up-regulated in response to negative growth signals, such as DNA damage (4, 5). Wild-type *p53* suppresses cell proliferation in cells with damaged DNA by arresting the cell cycle at the G_1-S border, thus allowing time to repair the DNA (1, 6). One mechanism by which *p53* arrests cell cycle progression is by induction of p21 expression. Induction of p21 results in the inhibition of cyclin/CDK-mediated phosphorylation of the retinoblastoma protein blocking the G_1 to S transition (7, 8). When DNA damage is severe, however, *p53* gene activation can also cause a cell to directly enter the apoptotic pathway (9).

Breast cancer is one of the few malignancies with an infrequent incidence of *p53* mutations (3). Because p21 acts downstream of *p53* in a tumor-suppression pathway, it seemed possible that tumors with wild-type *p53* genes would contain inactivating mutations in *WAF1*. A previous study of 36 invasive breast tumors revealed the presence of a single missense mutation in the *WAF1* gene (10). Toward a more rigorous analysis of the incidence of *WAF1* mutations in breast cancer, we analyzed the *WAF1* gene in 66 breast cancer samples and 19 cell lines from both the United States and Australia, and we also correlated these findings with the presence of mutations in the *p53* gene.

MATERIALS AND METHODS

Tissue and Cell Line Collection and Extraction. Surgical specimens of breast tumors were collected from Flinders Medical Center in Bedford Park (Adelaide, Australia) and Scripps Hospital (Encinitas, CA). Patients were classified as having stage 1 (tumor size, <2 cm; lymph node negative), stage 2 (tumor size, 2–5 cm), stage 3 (tumor size, >5 cm), or stage 4 (tumor any size with distant metastasis) breast cancer. All 33 tumors from the Australian patients were classified as stage 3. The 33 tumors from the United States patients were classified as stage 1 or 2. None of the patients had been treated with radiation or chemotherapy prior to resection.

A total of 19 breast cancer cell lines and two mammary epithelial cell lines were examined. Breast cancer cell lines BT474, BT549, HS578T, HS574BM, MDAMB157, MDAMB231, MDAMB 361, MDAMB 453, MDAMB 435, MDAMB 468, T47D, MCF-7, SKBR3, ZR75.1, and ZR 75.3 were grown under conditions recommended by the American

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[3] The abbreviations used are: PCNA, proliferating cell nuclear antigen; SSCP, single-stranded conformation polymorphism; CDK, cyclin-dependent kinase.

Table 1 *p53* and *WAF1* gene mutations in breast cancer from Australia and the United States[a]

	No. of tumors with *p53* mutations/total tested[b]				No. of tumors with *WAF1* mutations/total tested[c]		
Tumor no.	Exon	Codon	Nucleotide	Amino acid	Codon	Nucleotide	Amino acid
Group 1							
9	7	246	ATG→TG	Frameshift		WT	
11		WT			31[d]	AGC→AGA	Ser→Arg
17		WT			14	GGC→AGC	Gly→Ser
21		WT			31[d]	AGC→AGA	Ser→Arg
22	5	163	TAC→TAT	Tyr→Tyr	31[d]	AGC→AGA	Ser→Arg
	6	218	GTG→GAG	Val→Glu			
23		WT			31[d]	AGC→AGA	Ser→Arg
28		WT			31[d]	AGC→AGA	Ser→Arg
30	5	139	AAG→AAA	Lys→Lys		WT	
31	7	245	GGC→AGC	Gly→Ser		WT	
35	5	132	AAG→AAC	Lys→Asn	31[d]	AGC→AGA	Ser→Arg
36	5	165	CAG→TAG	Gln→Stop	9	CGT→TGT	Arg→Cys
	8	273	CGT→CCT	Arg→Pro			
Group 2							
5420		WT		Ref. 1	31[d]	AGC→AGA	Ser→Arg

[a] Incidence and nature of *p53* and/or *WAF1* gene mutations in a group of 33 breast cancer samples from Australia (group 1), 33 samples from the United States (group 2), and 19 breast cancer cell lines (group 3; not shown) from the United States.
[b] Group 1, 6/33 (18%); group 2, 6/33 (18%; Ref. 1); group 3 (cell lines; not shown), 9/19 (45%; Ref. 1).
[c] Group 1, 8/33 (24%); group 2, 1/33 (3%); group 3 (cell lines; not shown), 0/19 (0%).
[d] A naturally occurring polymorphism in the *WAF1* gene.

Type Culture Collection (Rockville, MD). Cell lines EP and MW were obtained from Dr. Renato Dulbecco (Salk Institute, San Diego, CA); cell lines 21PT and 21MT were kindly donated by Dr. Vimla Band (Tufts University, Boston, MA). Two mammary epithelial cell lines, MCF10A and HBL100, were obtained from the American Type Culture Collection.

Tissues and cell pellets were stored at −70°C, and DNA was extracted according to previously published methods (11).

SSCP Analysis of *WAF1* and *p53* Genes. The primer sets and amplification conditions for the SSCP analysis of the *p53* gene have been described in previous papers (12–14). The entire exon 2 and the 5′ region of exon 3, which includes both the initiation codon in exon 2 and the termination codon in exon 3 of the *WAF1* gene, were PCR amplified from genomic DNA in three fragments. To avoid amplification of possible pseudogenes, at least one primer in each primer set was designed (sequence of the primers provided by Bert Vogelstein, Johns Hopkins Oncology Center, Baltimore, MD) to anneal to intron sequences. Primer set 1 (fragment size, 263 bp): forward, 5′-CTTGTATCTCTGCTGCAGGC-3′ and reverse, 5′-CGTGGGAAGGTAGAGCTTG-3′; primer set 2 (fragment size, 278 bp): forward, 5′-ACTTCGCCTGGGA-GCGTGT-3′ and reverse, 5′-CGTGCACATGTCCGCACCT-3′; primer set 3 (fragment size, 112 bps): forward, 5′-TCCTCTTCTTCTTGGCCTGG-3′ and reverse, 5′AGGACTGCAGG-CTTCGTGT-3′.

SSCP analysis was performed according to previously published methods (14). Briefly, a 10-μl PCR was performed for 30 cycles in the presence of 1 μCi α^{32}P dCTP. The cycle progression for *WAF1* gene amplification was 94°C for 1 min, 58°C for 1 min (primer sets 1 and 2) or 1.5 min (primer set 3), and 72°C for 1 min. The PCR product was denatured in 95% formamide and 20 mM NaOH heated to 95°C for 5 min. The samples were run on an MDE gel (AT Biochem, Malvern, PA or FMC Bioproducts, Rockland, ME) in 0.6× Tris-borate EDTA buffer at 7 W for 12–15 h. When a bandshift was detected on the SSCP gel, the PCR product of that specific exon fragment was cloned and sequenced for *p53* or *WAF1* mutations.

Cloning and Sequencing of *WAF1* and *p53* Gene Exons. PCR-amplified DNA fragments were cloned into TA cloning vector (Invitrogen, San Diego, CA) and sequenced with Sequenase (Version 2.0; United States Biochemical, Cleveland, OH) according to the manufacturer's instructions.

Restriction Analysis of *WAF1* Arg Polymorphisms. To determine the incidence of the *WAF1* Arg allele (15–18), restriction enyzme analysis was performed on the 263-bp PCR-amplified product of *WAF1* gene (primer set I, see above) from DNA extracted from peripheral blood cells of 30 normal women (from the United States) and from 66 breast tumors from Australia and the United States. Ten μl of amplified products obtained by using primer set 1 were digested with 1 unit of the restriction enzyme *Hga*I (New England Biolabs, Beverly, MA) at 37°C overnight. Fifteen μl of the digest were electrophoretically fractionated on a 10% polyacrylamide gel and visualized under UV illumination after the gel was stained with ethidium bromide. *Hga*I digestion of the *WAF1*-Arg polymorph yielded products of 114 and 149 bps, whereas the *WAF1*-Ser polymorph remained undigested at 263 bps.

RESULTS

Mutations in the *WAF1* Gene Detected by SSCP and Confirmed by Nucleotide Sequencing. Nineteen human breast cancer cell lines, 33 primary tumors from United States patients, and all 33 tumors from Australian patients were examined for *WAF1* mutations by SSCP analysis of three PCR-amplified fragments. As shown in Table 1, none of the 19 breast cancer lines analyzed showed bandshifts indicative of sequence alterations. The normal SSCP profile for the *WAF1* gene in the breast cancer cell lines is consistent with a previous report in which *WAF1* gene mutations or deletions were not detected in

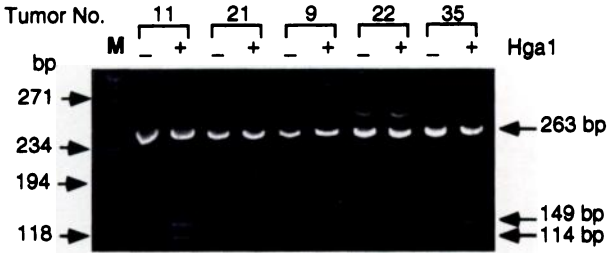

Fig. 1 Detection of *WAF1*-Arg polymorphism at codon 31. PCR-amplified DNA was digested with *Hga*I and fractionated by electrophoresis through a 10% polyacrylamide gel. Products were visualized by staining with ethidium bromide. A Phi X-*Hae*III (New England Biolabs) digest was used as a size marker (*M; left*). *Right,* sizes of the undigested DNA (−) and the *Hga*I-digested (+) PCR products. Presence of the uncut 263-bp fragment, as well as the 149- and 114-bp digestion products, in tumors 11, 21, 22, and 35 indicate the presence of *WAF1*-Ser/Arg alleles, whereas the lack of digestion in tumor 9 indicates the presence of *WAF1*-Ser/Ser alleles in this tumor.

human breast cancer cell lines (15, 19). Among the 66 primary breast tumors tested for *WAF1* sequence alterations, 9 (14%) exhibited bandshifts in SSCP gels. Seven (11%) of these shifts are identified by restriction enzyme and sequence analysis (Fig. 1) as an AGC→AGA (Ser→Arg) substitution in codon 31. This is a naturally occurring polymorphism that has been previously identified (16–18). The Australian cohort carried the polymorphism at a greater frequency than did the United States cohort (6 of 33 *versus* 1 of 33; Table 1).

Two other tumors in the Australian cohort exhibited a shift in *WAF1* SSCP analysis. Nucleotide sequencing of the PCR-amplified *WAF1* cDNA revealed that tumor 17 carried a G→A transition in codon 14 resulting in a replacement of glycine with serine, whereas tumor 36 carried a C→T transition resulting in an arginine to cysteine substitution in codon 9 (Fig. 2). Although *WAF1* sequence alterations are rare in tumors, the two base substitutions seen here have not been reported to be present in DNA of normal cells. The other known characteristics of the two tumors are that neither showed amplification of *HER2/neu* and that both arose in patients less than 55 years of age (49 and 33 years, respectively). Tumor 17 carried no *p53* mutations, was estrogen receptor and progesterone receptor positive, and metastasized to a single lymph node, and the patient relapsed within 5 years. Tumor 36 carried mutations in exons 5 and 8 of *p53*, was estrogen receptor and progesterone receptor negative, and did not metastasize to the lymph nodes or relapse within 5 years.

Distribution of the *WAF1* 31st Codon Polymorphism. Four of 30 (13%) DNAs from normal individuals and 7 of 66 (10%) breast cancer DNAs exhibited the presence of the *WAF1*-Arg polymorphism (15–18) as evidenced by the presence of the *Hga*I restriction cleavage site in the PCR product of the exon 2 sequences (Fig. 1).

SSCP Analysis for Mutations in the *p53* Gene. Mutations in the *p53* gene in breast cancer cell lines and breast tumors from the United States have been previously characterized (12–14). The 33 tumors from Australian patients were examined for mutations in *p53* exons 5–9. Six tumors (18%) exhibited mobility shifts by SSCP analysis (data not shown), which were confirmed by nucleotide sequencing (Table 1). Two of the six tumors carried mutations in two separate exons of the *p53* gene.

DISCUSSION

The nature of the key mutational events in breast cancers lacking *p53* mutations remain an open question. One area to begin looking for alternatives to *p53* mutations is in the genes encoding proteins associated with *p53*. Alternative target genes include: *mdm2* (20), *GADD45* (5), and *muscle creatine kinase* (21). The strongest known candidate, however, is *WAF1*, because it not only associates with p53 protein but is transcriptionally activated by *p53*. Furthermore, *p53* is known to play a major role in cell cycle regulation through its interaction with WAF1/p21/CIP1.

In this study, we investigated the potential role for *WAF1* mutations in breast cancer, particularly in those tumors that lack *p53* mutations. We determined that *WAF1* gene mutations are even less frequent than *p53* gene mutations in breast tumors of patients from the United States, as well as those from Australia. Although the frequency of the 31st codon polymorphism was greater in the Australian cohort than in the United States cohort, no significant correlation was found between the presence of the *WAF1* polymorphism and susceptibility to breast cancer.

The most common genetic alteration in the *WAF1* gene identified in this study was the base substitution of cytidine to thymidine in codon 31. This base transition, resulting in a serine→arginine amino acid change, is a commonly occurring polymorphism that is present in approximately 9% of the Caucasian American population (16–18, 22, 23). Our finding of a 13% incidence in normal individuals is well within the range of 10–18% in the United States. (16–18). The 18% incidence we found in the Australian cancer patients, although apparently much higher than the 3% incidence in the United States panel examined in this study, actually falls within the previously reported frequencies of United States levels. The polymorphism is not associated with a predisposition to nasopharyngeal cancer (16), ovarian cancer (24), colorectal cancer (25), or Burkitt's lymphoma (26), nor has a functional difference between the *WAF1*-Arg and *WAF1*-Ser genotypes been uncovered. The WAF1-Arg protein functions in an identical fashion to WAF1-Ser with respect to inhibition of CDC2 and cyclin A and growth of transfected cells (16). It appears from our study, as well as others, that the frequent presence of the *WAF1*-Arg polymorphism does not predict tumor susceptibility or aggressiveness.

Our study revealed that mutated *WAF1* genes are extremely rare in breast cancer. This also holds true for a variety of other tumor types, such including lung, ovarian, and pancreatic tumors (15–18, 22–26). In a prior study, direct sequencing of the PCR products from 36 invasive ductal breast cancers revealed a single C→T transition in codon 94 resulting in the substitution of an arginine by tryptophan in *WAF1*, an alteration that was not present in the normal DNA of the same patient (10). Functional analysis showed that this mutation reduced *WAF1*-mediated inhibition of CDKs but did not interfere with *WAF1* binding of cyclin-CDK or PCNA. Our finding that *WAF1* mutations were present in only 2 of 66 breast cancer samples and 19 breast cancer cell lines derived from populations from two different

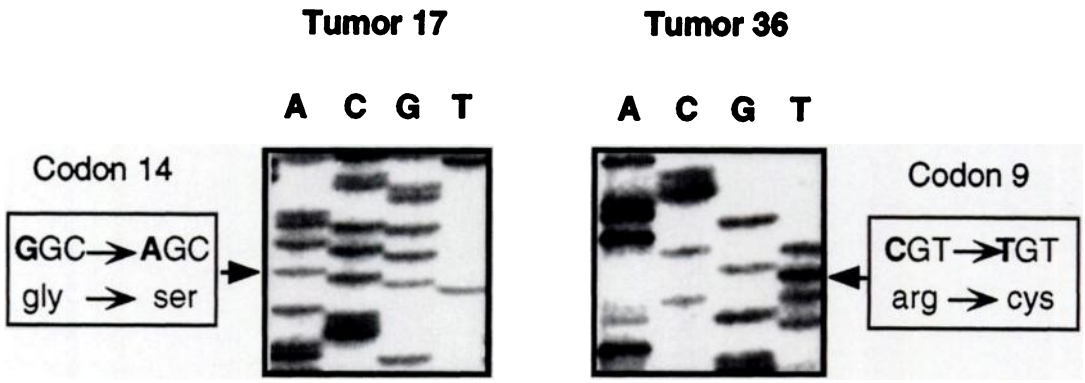

Fig. 2 Nucleotide sequence of *WAF1* in the two tumors with SSCP mobility shifts revealing novel missense mutations. The nucleotide sequence is read from the bottom of the gel to the top.

geographically locations is consistent with the previously reported low frequency of *WAF1* mutations. The rarity of mutations in *WAF1* might mean that: (*a*) inactivation of *WAF1* genes may be too deleterious for cell survival; (*b*) inactivation of *WAF1* can occur by other alterations at the transcriptional or translational level; or (*c*) mutations in *WAF1* do not lead to malignancy. Evidence against the first argument was provided by the recent finding that mice lacking *WAF1* undergo normal development (27).

In analyzing the potential mutations in *WAF1*, it is important to determine the locations at which genetic mutations would generate the greatest potential detriment. The *WAF1* gene structure consists of three exons of 68, 540, and 1600 bases coding for 164 amino acids (1). The transcription start codon resides in exon 2, and the termination codon resides just inside exon 3. WAF1 sequence analysis reveals a GC-rich 5′ end, a potential zinc finger domain (codons 13–41) and a region containing the nuclear localization signal (codons 140–163). We detected a mutation in the GC-rich region at the 5′ end of the coding sequence and in codon 14, which is of the potential zinc finger domain. The functional significance of these regions in the WAF1 protein has not yet been experimentally elucidated. It is clear that they are not involved in binding to PCNA, Cdk, or DNA (28–31). A combination of mutational studies in cancer and functional studies will further define the important domains of the p21/WAF1/CIP1 protein.

The *p53* gene has been firmly established as a mutational target in a small but notable proportion of breast cancers (12, 13, 32–34). The *p53* mutation frequency in breast cancer averages around 20% with a range of 4–46% (3). In the panel of tumors selected for this study, we detected the same mutational frequency in the Australian tumor set (18%) as in breast tumors from the United States. Therefore, the incidence of *p53* mutations in breast tumors found in this study is in agreement with previous findings.

In conclusion, this investigation has identified two novel mutations in the *WAF1* gene. However, we find that mutations in *WAF1* and *p53* genes are generally rare in sporadic human breast tumors (including late stage tumors), in tumors derived from disparate populations, and in breast tumor cell lines. Although these findings argue against a role for *WAF1* gene mutations in the causation of breast tumors carrying wild-type *p53* genes, they emphasize the need for further investigation into the genetic abnormalities that lead to breast cancer.

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