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Preferential Loss of Expression of *p16^{INK4a}* Rather Than *p19^{ARF}* in Breast Cancer[1]

Andrew J. Brenner, Abhaya Paladugu, Hui Wang, Olufunmilayo I. Olopade, Martin H. Dreyling, and C. Marcelo Aldaz[2]

Department of Carcinogenesis, The University of Texas M. D. Anderson Cancer Center, Research Division, Smithville, Texas 78957 [A. J. B., A. P., H. W., C. M. A.], and University of Chicago Medical Center, Chicago, Illinois 60637 [O. I. O., M. H. D.]

ABSTRACT

The tumor suppressor *p16^{INK4a}* has been shown to be inactivated in numerous cancer lines and primary tumors. Recently, we reported loss of heterozygosity of the region in which *p16^{INK4a}* is located in more than one-half of primary breast tumors. However, mutational analysis of these same tumors revealed mutation of *p16^{INK4a}* to be infrequent. Other possible modes of inactivation, such as *de novo* methylation and homozygous deletion, have since been shown to occur in numerous neoplasias. Furthering the complexity of this locus, a transcript overlapping the *p16^{INK4a}* coding sequence and encoding a novel peptide with growth-suppressive activity, *p19^{ARF}*, has been described. To clearly elucidate the target of aberrations affecting this subchromosomal region and approximate frequency in breast cancer, we performed a comprehensive study including *p16* deletion analysis by means of interphase chromosomal fluorescence *in situ* hybridization, methylation analysis of the first exon encoding *p16^{INK4a}* (exon 1α), mutational analysis of exon 1β by single-strand conformational polymorphism analysis of *p19^{ARF}* transcripts, and expression of both α and β transcripts by reverse transcription PCR. Homozygous deletion of *p16*, as determined by interphase chromosomal fluorescence *in situ* hybridization, was observed in 3 of 18 (17%) tumors analyzed, whereas *de novo* methylation of exon 1α was observed in an additional 17% (4 of 23). Reduced expression of *p16^{INK4a}* was observed in 11 tumors (48%), including all those in which homozygous deletion or complete methylation was observed. No mutations of exon 1β were detected, and expression of its transcript was variable, with 13% demonstrating decreased expression and 17% demonstrating overexpression. These results further support *p16^{INK4a}* as a target of inactivation in the 9p21 region for breast cancer.

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[2] To whom requests for reprints should be addressed, at Department of Carcinogenesis, The University of Texas M. D. Anderson Cancer Center, Science Park, Research Division, Smithville, TX 78957. Phone: (512) 237-2403; Fax: (512) 237-2475.

INTRODUCTION

Chromosomal subregion 9p21 has been shown to undergo hemizygous and homozygous deletion in a variety of tumor types (1–3). Recently, we have reported hemizygous loss to occur frequently in breast cancer (58%) as well (4). Previous analysis of this region had shown it to contain *p16^{INK4a}*, an inhibitor of cyclin-dependent kinases, commonly referred to as *p16* (5, 6). Nevertheless, when we subjected these same tumors to mutational analysis of *p16*, we observed few tumors with sequence alterations of consequence, thus suggesting that *p16* may not be the target of such anomalies in breast cancer (4). However, recent reports have indicated that mutation may not be the primary mechanism of inactivation of the *p16* gene in many tumor types (7–12).

Recently an alternative transcript encoded from the same second and third exons encoding *p16* and using the same splice site, but with a separate promoter and alternative first exon, now referred to as exon 1β, has been described (13, 14). This alternative transcript is abundant in various tissue types (13, 14) and is translated in the mouse from an alternative reading frame, resulting in a protein of 19 kilodaltons with cell cycle-arresting capacity, now termed p19^{ARF} (15). Thus, the *p16* locus appears to be complex, with two overlapping transcripts translated from distinct reading frames, resulting in two polypeptides, p16^{INK4a} and p19^{ARF}, each able to induce cell cycle arrest. Because the transcripts of these two polypeptides partially overlap, it may be that alterations that affect one may also affect the other. Therefore, the possibility exists of p19^{ARF} being a protein with tumor-suppressive function being targeted for inactivation as well. A comprehensive analysis of the aberrations affecting the *p16* and *p19* genes and expression of transcripts *in vivo* should help clarify these issues.

To help elucidate the role of aberrations affecting these two genes in breast cancer, we have performed a comprehensive analysis of inactivation and expression in a series of primary breast carcinomas. To that end, we performed IC-FISH[3] deletion analysis of the *p16* region, methylation analysis of the 5′ region of the primary first exon, SSCP analysis of the alternative exon 1β transcript, and expression of both transcripts (α and β) by semiquantitative RT-PCR. Taken together with previously determined microsatellite polymorphism LOH analysis of 9p21 and *p16* mutational analysis in these same tumors (4), we believe we have obtained a more complete account of p16^{INK4a} and putative p19^{ARF} involvement in breast tumorigenesis.

[3] The abbreviations used are: IC-FISH, interphase chromosomal fluorescence *in situ* hybridization; SSCP, single-strand conformational polymorphism; RT, reverse transcription; LOH, loss of heterozygosity; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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MATERIALS AND METHODS

Tissue Samples, DNA Extraction, and RNA Extraction. Normal and tumor breast samples were obtained from the Cooperative Human Tissue Network. All samples were invasive ductal carcinomas with the exception of cases 24 (ductal carcinoma *in situ*) and 32 (mucinous adenocarcinoma). Samples were snap frozen with liquid nitrogen less than 1 h after surgery. Regions dense in tumor cells were identified by visual inspection and comparison with H&E slides as necessary. Total genomic DNA was isolated using phenol:chloroform:isoamyl (25:24:1) in Phase Lock gel tubes (5 Prime → 3 Prime, Inc., Boulder, CO) according to standard protocol and precipitated with 2.5 volume ethanol. Total RNA was isolated using an RNEasy Total RNA kit (Qiagen, Chatsworth, CA) as per the manufacturer's instruction.

IC-FISH. Probe pHUR98, a variant satellite 3 sequence that specifically hybridizes to the heterochromatic region of chromosome 9, was used to assess chromosome 9 copy number. p16cos is a contig of eight cosmids encompassing a 250-kb region around *p16*, obtained by screening a flow-sorted chromosome 9 library (10) and used to determine *p16* copy number. Probes were amplified and labeled by sequence-independent amplification (16) and either biotin-11-dUTP (Enzo Diagnostics) labeled (centromeric probe pHUR98) or digoxigenin-11-dUTP (Boehringer Mannheim) labeled (p16cos contig). Dual-color chromosomal FISH was performed as described previously (10). Biotinylated probes were detected with Texas red-avidin (Vector Laboratories), and digoxigenin-labeled probes were detected with FITC-conjugated antibodies (Boehringer Manheim). Gray-scale images corresponding to each fluorochrome were captured from tumor cell interphases selected at random, using a Photometrics (Tucson, AZ) CCD cooled camera. Pseudocolor composite images were analyzed using Oncor (Gaithersburg, MD) Image software. A total of 150 intact tumor cells was analyzed by three independent observers (A. B., A. P., and C. M. A.). Cells with no visible p16cos signal were interpreted as nullizygous for p16 and labeled as total deletions, whereas cells displaying a single copy of the p16 region as well as cells with relative deletion (fewer copies of the p16 region than centromeric pHUR89) were labeled as partial deletions. To avoid overinterpretation of incomplete hybridization, based on analysis of normal human lymph nodes, tumor cell populations were not included in scoring of deletions unless present at ≥10%.

Southern Hybridization. Methylation analysis was performed as described previously (7, 17). Briefly, 10 μg of genomic DNA were digested with a flanking site enzyme (either *Eco*RI or *Hin*dIII) and a methylation-sensitive endonuclease (*Sac*II, *Sma*I, or *Eag*I), ethanol precipitated, resuspended, and resolved in a 1% agarose gel overnight. DNA was transferred to a Zeta-Probe nylon membrane (Bio-Rad, Richmond, CA) and hybridized with a 340- or 280-bp [α-^{32}P]dCTP random prime-labeled PCR fragment including exon 1α, as described (7, 17). Autoradiographs were obtained following 2–4 days of exposure.

RT-PCR. Five μg of total RNA were used for first-strand cDNA synthesis with Superscript II reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) as per the manufacturer's instructions. Following RT, each sample was subjected to analysis of GAPDH levels as control of mRNA quantity by PCR amplification using a GAPDH Positive control primer set (Stratagene, La Jolla, CA). RT-PCR of both *p16* α and β transcripts was performed as described previously (13). An initial experiment was performed to validate the quantitative nature of the RT-PCR, as reported previously (13), and was found to concur (data not shown). Mean normal expression was obtained by analysis of four normal reduction mammoplasty breast samples. Signal intensities in all cases were analyzed and quantified with a Molecular Dynamics PhosphorImager. A relative value of 1.0 was assigned to the ratios of α:GAPDH and

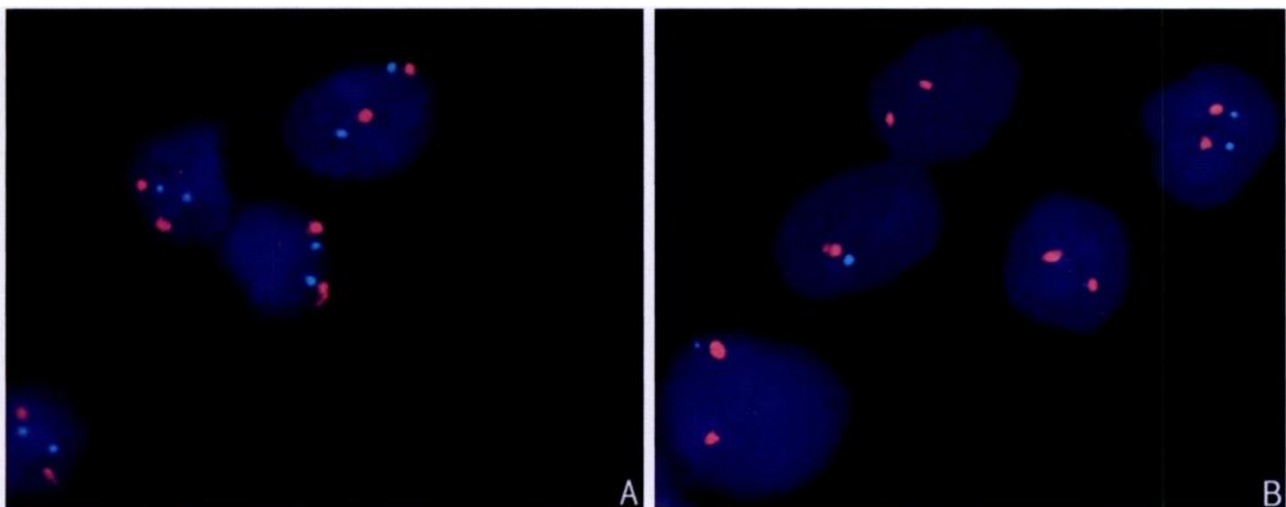

Fig. 1 Bi-color chromosome FISH performed with chromosome 9 centromere-specific probe (*red*) and *p16*-specific probe (*green*). *A*, representative interphase nuclei from a normal human lymph node displaying two alleles of both the centromeric region of chromosome 9 (*red*) and the p16cos region (*green*). *B*, representative interphase nuclei from human breast carcinoma sample 28. Note the heterogeneity of the tumor with cells showing no p16cos (*green*) signal, one p16cos signal, or two p16cos signals.

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β:GAPDH based on normal mean, and subsequent samples were normalized accordingly. All experiments were performed in triplicate, and SDs were calculated.

SSCP. Amplification of β transcripts was performed as described above. RT-PCR products were diluted 2:1 in denaturing loading buffer (95% formamide, 10 mM NaOH, and 0.1% xylene cyanol) and resolved in 6% polyacrylamide using two conditions: 10% glycerol and 1× Tris-borate EDTA at 16 W for 16 h; and 5% glycerol and 0.5× Tris-borate EDTA at 8 W for 8 h.

RESULTS AND DISCUSSION

***p16^INK4a^* Locus Copy Number.** The 9p21 chromosomal subregion has been shown to undergo LOH in a variety of neoplasias (1, 18, 19). Although the $p16^{INK4a}$ gene is known to be located within this region, mutational analysis of the $p16^{INK4a}$ gene in breast tumors revealed infrequent mutations (<5%; ref 4). To determine whether these tumors may have alternatively incurred homozygous deletion of the *p16* region, and to what extent, we performed dual-color IC-FISH using the 250-kb p16cos contig and a centromeric probe for chromosome 9 copy number (Fig. 1). We observed total or partial deletion of the *p16* chromosomal region in 61% (11 of 18) of breast tumors. This result is comparable to our aforementioned analysis of the same tumor set by PCR-based microsatellite length polymorphism analysis (4). Specifically, three cases (17% of the total) displayed tumor cell subpopulations with total deletion of the p16cos region. Subsequent analysis by RT-PCR showed low to absent expression in both of the two cases for which sufficient material was available. However, although partial deletion was more common (11 cases) than total deletion, subsequent expression analysis did not show a strong association between partial deletion and loss of expression (Table 1). Tumor cell populations displaying hyperdiploidy of chromosome 9 were observed in eight (45%) of the tumors analyzed. No tumors showed evidence of significant chromosome 9 monosomy.

To our knowledge, this is the first report of homozygous deletion of the *p16* region in primary breast carcinomas through the use of *in situ* hybridization. Although two previous studies have been conducted on breast carcinomas by Southern analysis, cumulatively no homozygous deletions were reported of the 21 breast tumors analyzed (9, 20). Because breast cancer samples may also contain a significant portion of normal, nonneoplastic stromal or epithelial cells or heterogeneous tumor cell populations, it may be that Southern analysis is not of sufficient sensitivity for determinations of homozygous loss. However, another study based on microsatellite analysis reported homozygous loss at a frequency comparable to that reported here (21).

Hypermethylation of *p16^INK4a^* Exon 1α. Exon 1 of the $p16^{INK4a}$ gene contains a documented CpG island, which has been shown to be unmethylated in normal tissue and hypermethylated in certain tumor types at varying incidence (7–9). To establish the methylation status of the 5′ region, total genomic DNA was digested with a combination of a flanking site endonuclease and a methylation sensitive endonuclease, as described previously (9). Twenty-three tumors were analyzed, of which patterns consistent with partial or total methylation were observed in four (17%; Table 1 and Fig. 2). Two tumors (tumors 30 and 41) showed methylation with multiple restriction enzymes (*Sac*II and *Sma*I), whereas two others (tumors 16 and 44) revealed methylation with only one enzyme (*Eag*I and *Sac*II, respectively). Of those tumors showing methylation, three (tumors 16, 30, and 41) displayed patterns consistent with methylation of all possible endonuclease sites in that region, whereas one (tumor 44) displayed methylation of a single site. The remaining 19 tumors revealed no pattern consistent with hypermethylation. These results are consistent with a previous report of methylation in primary tumors of the breast, although the frequency observed here (17%) is somewhat lower than the frequency reported previously (31%) by Herman *et al.* (9).

Expression of *p16^INK4a^* α and β Transcripts. As previously suggested by Stone *et al.* (13), the similarity in size and sequence of the α and β transcripts may have complicated previous efforts to measure *p16* RNA levels by Northern blot (13) in different neoplasias. Only an analysis of transcripts using the unique sequences of exon 1α would be able to asses the true levels of *p16* expression. Additionally, because inactivating events that target *p16* may also affect the alternative β transcript, and because we know the alternative β transcript to

Table 1 Analysis of $p16^{INK4a}$ and $p19^{ARF}$ in breast cancer

Tumor no.	Deletion status (% of cells)[a] Total	Partial	Methylation[b]	α (p16) expression[c]	β (p19) expression[c]
6	68	11	–	↓↓	↓
28[d]	44	15	–	↓↓	N
48[d]	10	50	–	nd	nd
16	nd	nd	+	↓↓	↓↓
30[d]			+	↓	N
41[d]	nd	nd	+	↓↓	↑
14		11	–	↓↓	N
18			–	↓↓	N
4	nd	nd	–	↓↓	N
8		44	–	↓	N
10			–	↓	N
22[d]		47	–	↓	N
26[d]	nd	nd	–	N	↓
44[d]	nd	nd	+/–	N	N
11[d]		36	nd	N	N
24[d]			–	N	N
32[d]			–	N	N
34[d]			–	N	N
36[d]			–	N	N
38[d]	nd	nd	–	N	N
50[d]		23	–	N	N
20[e]		28	–	N	↑
2		23	–	↑	↑
54		23	–	↑	↑

[a] Determined by the relative signal of p16cos to chromosome 9 centromeric probe in IC-FISH analysis. Tumor cell populations were not included in scoring of deletions unless present at ≥10% (see "Materials and Methods"). nd, not determined.

[b] Methylation status determined by Southern analysis as described in "Materials and Methods." –, no methylation; +, methylation; +/– partial methylation.

[c] Expression determined by RT-PCR analysis as described in "Materials and Methods." ↓↓, <10% of normal mean expression; ↓, <30% of normal mean expression; ↑, >300% of normal mean expression; N, normal expression.

[d] Wild-type p16 exon 2 (4).

[e] Polymorphism base 140, exon 2 (4).

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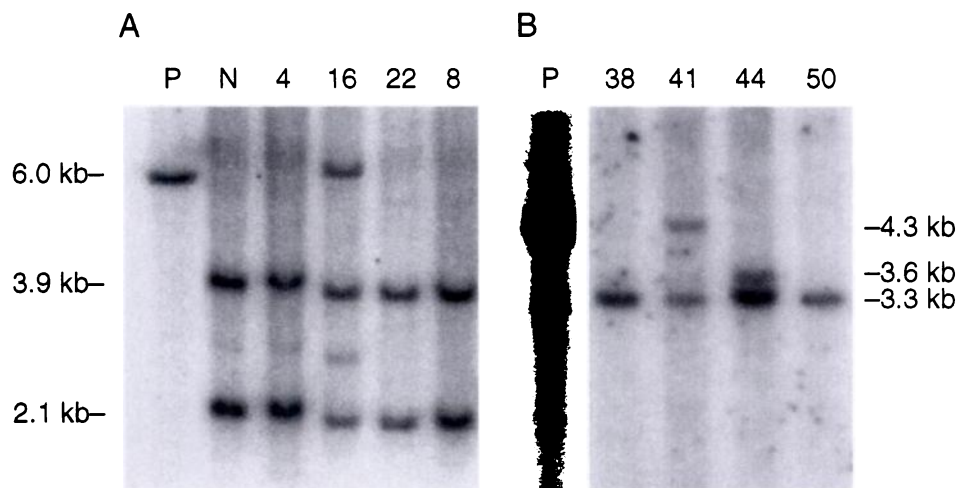

Fig. 2 Representative Southern analysis of *p16* exon 1α in primary breast carcinomas with methylation-sensitive restriction enzymes. *A*, analysis of methylated line T47D (*P*, positive control; Ref. 9), normal breast DNA (*N*), and four breast carcinomas with *Hin*dIII and *Eag*I reveal methylation of sample 16. *B*, analysis of methylated line T47D (*P*) and four breast carcinomas with endonucleases *Eco*RI and *Sac*II reveal complete methylation of tumor 41 and partial methylation of tumor 44.

encode $p19^{ARF}$ and to have growth-suppressive effects in murine cells *in vitro*, it would be advantageous to evaluate both *p16* and alternative β transcript expression independent of one another.

Expression levels of *p16* α and β transcripts in 23 tumors were determined by RT-PCR analysis. Expression levels of each transcript were subsequently compared with mean normal expression of a panel of four normal breast samples. Expression of both the *p16* primary α and alternative β transcripts in breast tumors was varied (Table 1 and Fig. 3, *A* and *B*). Six of 23 (26%) showed expression of $p16^{INK4a}$ at levels less than 10% of the normal mean, whereas another 5 (22%) revealed levels of expression from 10 to 30% of the normal mean (*i.e.*, greater than 70% reduction in normal expression; see Fig. 3). Loss of expression in many of these tumors can be accounted for by either hypermethylation (tumors 16, 30, and 41) or homozygous deletion (tumors 6 and 28). However, inactivation by either of these mechanisms was not observed in some cases in which loss of expression was observed, indicating that other modes of inactivation could be operative. Moreover, a previous report of $p16^{INK4a}$ expression by immunohistochemistry suggested loss of expression in as many as 65% of breast tumors (22), indicating that inactivating events might be possible at a posttranscriptional stage as well. We also observed that two additional carcinomas, tumors 2 and 54, displayed what appears to be overexpression of $p16^{INK4a}$, concomitant with β overexpression. Previous analyses in numerous lines have indicated that the overexpression of *p16* can be associated with retinoblastoma protein inactivation (23). However, no precedent of this association has been described *in vivo*. Nonetheless, such overexpression may be deemed aberrant.

Analysis of β transcripts showed great variability in expression, with apparent overexpression to be as prevalent as lack of expression. One of the 23 tumors analyzed revealed undetectable levels of expression (tumor 16). Two additional carcinomas (tumors 6 and 26) showed expression at less than 30% of the normal mean. Incidentally, the two cases in which the lowest expression of β was observed (tumors 6 and 16), both displayed α loss, and by distinct mechanisms. Loss of expression in tumor 6 appeared to be through homozygous deletion, whereas loss of expression in tumor 16 appeared to be through methylation and LOH (4). However, methylation of exon 1α only explains loss of expression of the primary α transcript. Perhaps in some cases, such as tumor 16, the methylation of the 5′ region of exon 1α is indicative of the hypermethylation of the entire locus, and as such, the 5′ region of exon 1β also could be hypermethylated. Because the 5′ region of exon 1β from −180 to +266 bp contains 70% GC content and a CG:GC ratio of 0.71, thus defining a CpG island, this probability exists. Of additional interest, four tumors (tumors 2, 20, 41, and 54) showed considerably high levels of β ($p19^{ARF}$) expression between 3- and 5-fold greater than the normal mean. What level of increased expression can be considered significant and the possible implications of such overexpression have yet to be determined.

The possibility of $p19^{ARF}$ being tumor suppressive in function has been postulated previously (13, 15). However, this has not yet been shown, and an analysis of the expression of this transcript in neoplastic tissue was not reported previously. Previous attempts to address this issue through sequence analysis of exon 1β in other tumor types revealed no mutations (14). In this report, we addressed the issue of possible β inactivation in breast cancer by performing SSCP analysis of the exon 1 region of β transcripts in all 23 tumors for which we obtained expression data and found no evidence of mutation in any of the tumors (data not shown). Although our own previous analysis (4) of exon 2 in breast tumors revealed three mutations of 21 tumors affecting the amino acid sequence for the β transcript (CGA-GGA, codon 87; GCA-GTA, codon 96; and CGC-CAC, codon 161), only one of these mutations was found in a region conserved in both mice and humans, and another was a frequently reported polymorphism. Furthermore, we have now shown that apparent loss of expression of the β ($p19^{ARF}$) transcript is primarily observed in those breast tumors in which $p16^{INK4a}$ expression is compromised. Taken together, this information indicates that point mutation or loss of expression is not common for the β transcript, and that there is no evidence to suggest a tumor-suppressive role for the β transcript in breast carcinogenesis. However, the reason for and possible conse-

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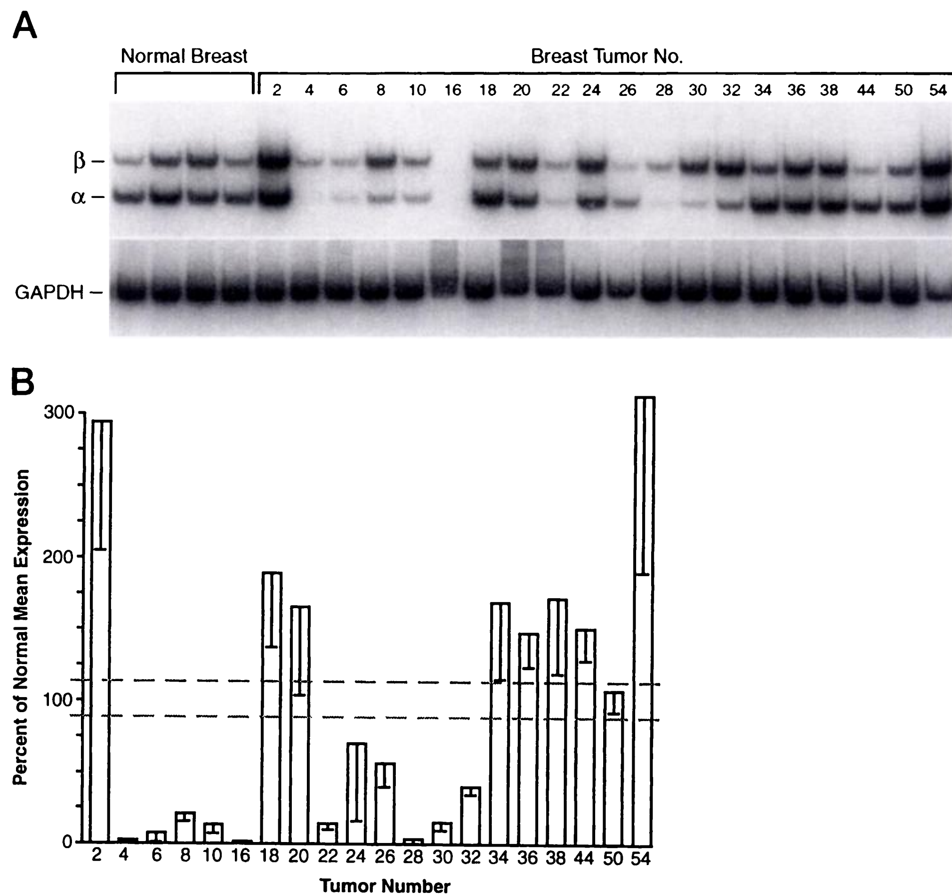

Fig. 3 Expression analysis of the α ($p16^{INK4a}$) and β ($p19^{ARF}$) transcripts in breast cancer by RT-PCR. *A*, autoradiograph of a representative analysis of α and β transcripts in 4 normal breast samples and 20 carcinomas of the breast. *B*, GAPDH-normalized expression levels of the α transcript as determined by triplicate analysis in 20 tumors (see text). *Dashed line*, normal mean SD; *bar*, sample SD.

quences of the observed overexpression of the β transcript in some breast tumors are unclear. Further experiments are needed to address these issues.

Nevertheless, comprehensive analysis of homozygous and hemizygous deletion, methylation, mutation, and expression suggest that the tumor suppressor $p16^{INK4a}$ is cumulatively affected in approximately 40–50% of the breast carcinomas analyzed. This rate of inactivation of $p16^{INK4a}$ and lack of inactivation of the β transcript implicate $p16^{INK4a}$ involvement in the tumorigenesis of the breast at a rate greater than or equal to that reported previously for any other tumor suppressor gene in sporadic breast cancer.

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