**INa Gene Expression and Methylation in Primary Breast Cancer: Overexpression of p16INK4a Messenger RNA Is a Marker of Poor Prognosis**


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**ABSTRACT**

Frequent deletions or mutations of the INK4 gene, which encodes the cyclin-dependent kinase 4 inhibitor p16INK4a, have been documented in various human cancers, but little is known about the role of this tumor suppressor gene in primary breast cancer. We examined p16INK4a mRNA expression and its relationship with cyclin D1 and estrogen receptor (ER) expression in 314 primary breast cancers using Northern blots probed with a p16 exon 1-specific cDNA. Tumor samples overexpressing p16INK4a were predominantly ER negative with low levels of cyclin D1. Cyclin D1 and ER mRNA levels in the high p16INK4a expressers were significantly lower than those in the remainder of the population (P = 0.0001). Furthermore, the mean p16INK4a mRNA level in the ER-negative tumors was significantly higher than that in the ER-positive group (P = 0.0001). Because the INK4 gene is frequently inactivated by de novo methylation, we investigated the frequency of INK4a exon 1a methylation in a subset of 120 primary breast cancers using methylation-specific PCR; 24 of these were methylated. These findings indicate that high expression of p16INK4a and reduced expression due to de novo INK4a methylation are frequent events in primary breast cancer. In a subset of 217 patients for whom detailed clinical data were available, high p16INK4a mRNA expression was associated with high tumor grade (P = 0.006), ≥ 4 axillary lymph node involvement (P = 0.004), ER negativity (P = 0.0001), and increased risk of relapse (P = 0.006). The significant negative correlation between p16INK4a and ER gene expression raises issues regarding their functional interrelationships and whether high p16INK4a expression may be associated with a lack of hormone responsiveness in breast cancer.

**INTRODUCTION**

p16INK4a and other members of the INK4 family of Cdk inhibitors inhibit the G1 cyclin D-dependent kinases, Cdk4 and Cdk6, which phosphorylate pRb and facilitate entry into S phase (1, 2). The fact that p16INK4a can block G1-S-phase progression and that mutant p16INK4a proteins are nonfunctional in cell cycle arrest or Cdk inhibition suggests that p16INK4a plays an important role in negative growth control (3, 4). Moreover, the finding that the INK4a gene is frequently deleted in cancer cell lines (5, 6) indicates that it may be a tumor suppressor gene. This was subsequently confirmed in a p16INK4a knockout mouse model in which there is direct evidence that p16INK4a deficiency facilitates tumor development (7). Furthermore, deletions and mutations of the INK4a gene develop in early lesions of Barrett’s esophagus, head and neck cancer, and bladder cancer (8–11), suggesting that molecular alterations leading to p16INK4a inactivation occur relatively early during carcinogenesis at some tissue sites.

The absence of p16INK4a expression is seen predominantly in cells that retain wild-type RB (12). However, p16INK4a is overexpressed in cancer cell lines and tumors in which pRb is dysfunctional (13–16), providing evidence for a negative feedback loop in which the functionally inactive pRb fails to sequester transcription factors, which, in turn, induce INK4a gene expression. There is accumulating evidence to suggest that expression of p16INK4a and pRb is mutually counterbalanced to maintain growth-inhibitory activity in the cyclin D1-Cdk4-p16INK4a-pRb pathway of cell cycle control. In cultured cells, pRb represses transcription of the INK4a gene (17), whereas expression of p16INK4a induces transcriptional down-regulation of the RB gene (18). In the event of inactivating mutations or deletion of one of these tumor suppressor genes, the other would be overexpressed (17, 18); however, high expression of either pRb or p16INK4a alone is incapable of inhibiting cell cycle progression (19).

p16INK4a and p15INK4b are colocalized to chromosome 9q21.
9p21, a locus commonly deleted in several human cancers. The INK4a gene has a complex structure. Transcription of the INK4a gene can yield two distinct transcripts (α or β mRNAs) coding for two functionally distinct proteins, p16INK4a and p19ARF (for alternative reading frame). The two transcripts have the same exons 2 and 3 but contain a different exon 1, designated exon α and exon β (20–23). Whereas the α transcript is selectively expressed in some tissues in humans as well as mice, the β transcript is ubiquitously expressed. Given the dissimilarity in structure with other known Cdk inhibitors and the failure to coprecipitate p19ARF with Cdkc2, Cdkc2, Cdk4, Cdk6, cyclin D2, cyclin D3, cyclin E, and cyclin A, p19ARF is not a direct Cdk inhibitor. However, ectopic expression of p19ARF in cells with homozygous deletion of INK4a induces cell cycle arrest in both the G1 phase and G2-M phases of the cell cycle with a concomitant loss of cells in S phase (22). More recent work has indicated that p19ARF interacts with MDM2, promotes MDM2 degradation, and, in turn, stabilizes p53 (24, 25). Thus both p16INK4a and p19ARF induce cell cycle arrest at apparently different points in the cell cycle and via distinct mechanisms; consequently, deletion of INK4a impairs both the pRb and p53 tumor suppressor pathways.

Inactivation of INK4a occurs frequently in a wide spectrum of sporadic primary cancers and familial melanoma. Mutation or homozygous deletion of INK4a occurs with a frequency ranging from approximately 20% in sporadic melanoma, non-small cell lung cancer, head and neck cancer, esophageal cancer, and malignant mesothelioma to 30% in transitional cell cancer of the bladder, 35% in gliomas, and 50% in pancreatic cancer and squamous cell carcinoma of the bladder (6, 26–29). However, unlike the situation in cancer cell lines, homozygous deletion and mutation of INK4a are very rarely observed in primary breast cancers (26, 30, 31). DNA methylation of the human INK4a gene is associated with gene silencing and hence inactivation of INK4a in some human cancers including head and neck, lung, brain, colon, esophageal, and bladder cancers and also in a small series of breast cancers (32, 33).

In general, p16INK4a inactivation is associated with a more aggressive phenotype and worse prognosis in a wide range of neoplasms including pancreatic carcinoma, malignant melanoma, glioma, leukemia, non-Hodgkin’s lymphoma, and non-small cell lung cancer (34–45). In breast cancer, one published study (46) reported an association between LOH at 9p21–22 and worse prognostic features including high S-phase fraction, aneuploidy, and large tumor size (≥2 cm), although no association with patient survival was demonstrated in a short period of 3 years median follow-up. Conversely, another smaller study (47) failed to demonstrate a relationship between LOH at 9p21–22 and other clinicopathological parameters in 68 breast cancers. The only study available in the literature specifically examining the prognostic significance of p16INK4a in breast cancer reported that poor outcome was associated with high expression of p16INK4a as assessed by strong immunohistochemical staining (16).

Given the paucity of data on the prognostic significance of the tumor suppressor gene INK4a in breast cancer, a study of p16INK4a mRNA expression, including its relationship with cyclin D1 and ER status, was initiated in a series of tumors from 314 patients. The relationship between p16INK4a and various clinicopathological features and clinical outcome was studied in a subset of 217 patients. Furthermore, the frequency of INK4a methylation was also investigated using methylation-specific PCR in a subset of 120 samples.

MATERIALS AND METHODS

Clinicopathological Features. The clinical details of the series of patients included in this study have been described previously (48–50). In brief, Northern blots containing total RNA extracted from breast cancers of 364 patients who underwent surgery at the Nottingham Breast Unit during the period between February 1987 and December 1993 were probed with p16INK4a exon 1α cDNA. Of the 314 breast cancers evaluable for p16INK4a mRNA expression, full clinical follow-up data were available on 217 patients with stage I or II disease. All patients under 70 years of age underwent axillary LN sampling with either simple mastectomy or wide local excision followed by adjuvant postoperative radiotherapy. Adjuvant chemotherapy with cyclophosphamide, methotrexate, and 5-fluorouracil or tamoxifen was introduced from 1989, based on the Nottingham Prognostic Index (51), age, and ER status.

Data on cyclin D1, ER, and 36B4 mRNA expression were available from previous studies on this series of breast cancers (49, 50).

Breast Cancer Cell Lines and cDNA Probes. The sources of the breast cancer cell lines were as described below. BT-20, BT-483, BT-549, DU-4475, HS-578T, MDA-MB-134, MDA-MB-175, MDA-MB-361, MDA-MB-436, MDA-MB-453, MDA-MB-468, SK-BR-3, and ZR-75-1 were obtained from the American Type Culture Collection (Manassas, VA). HBL-100, MCF-7M, MDA-MB-157, MDA-MB-231, MDA-MB-330, and T-47D were obtained from the EG & G Mason Research Institute (Worcester, MA). The full-length 960-bp p16INK4a cDNA was provided by Dr. David Beach (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

A 340-bp p16INK4a exon 1α fragment was generated by PCR from human PBL DNA using exon 1α primers 5'-GAAAGAGGAGGGCTG and 5'-GAAAGAGGAGGCTG and 5'-GCGCTACCTGATTC-GAAAGAGGAGGGCTG and 5'-GCGCTACCTGATTC-GAAAGAGGAGGGCTG. PCR reactions were performed in a total volume of 50 μL. The reaction contained 5 μL of 10× PCR buffer [pH 8.3; 100 mM Tris-HCl, 500 mM KCl, 15 mM MgCl2, and 0.01% gelatin], 10 mmol of deoxyxynucleotide triphosphate, 10 pmol of each primer, 0.1 μg of DNA template, 3.6% formamide, and 5 units of Taq polymerase (Boehringer Mannheim, Mannheim, Germany). PCR reactions were overlaid with mineral oil. The cycling parameters included an initial denaturing step at 94°C for 4 min, followed by 92°C for 1 min, 52°C for 30 s, and 72°C for 30 s for 30 cycles, and a final elongation cycle of 72°C for 5 min. The amplified PCR product was purified through two Sepharose 6 L CB (Sigma Chemical Co., St. Louis, MO) columns equilibrated with 1× TE buffer [pH 7.4; 10 mM Tris and 1 mM EDTA] and centrifuged for 5 min at 600 × g. The 340-bp p16INK4a exon 1α cDNA was validated by sequencing.

Analysis of p16INK4a mRNA Expression. The details of total RNA extraction from the primary breast cancer samples and Northern blotting to determine the expression of cyclin D1, ER mRNA, and the ribosomal protein 36B4 as a control for RNA loading were described previously (49, 50). The filters...
were strip washed in 0.1% SSC and 0.1% SDS at 100°C for 5 min and repropbed with the specific p16INK4a exon 1α PCR product. Each filter contained four control cell lines for normalization between the 22 filters. The ratio of p16INK4a exon 1α:36B4 mRNA signal intensity for each sample was normalized to that of HBL-100, which was defined arbitrarily as 10 to yield “relative expression of p16INK4a mRNA.”

DNA Methylation Assay. Methylation of the INK4a gene in breast tumors and cell lines was detected by methylation-specific PCR (52) using the CpG WIZ Methylation Assay [Oncor, Gaithersburg, MD]. One μg of tumor or cell line DNA was modified by sodium bisulfite according to the protocol and subsequently purified by ethanol precipitation. All unmethylated cytosines were converted to uracils, whereas 5-methylcytosines remained unaltered. The methylation status of the treated DNA was then determined by PCR amplification using specific primers within the promoter region. The reaction contained 2.5 μl of 10× PCR buffer [20 mm Tris-HCl (pH 7.5), 100 mm KCl, 1 mm DTT, 0.1 mm EDTA, 15 mm MgCl₂, 0.5% Tween 20, 0.5% NP40, and 50% glycerol], 2.5 μl of 2.5 mm deoxynucleotidetriphosphate mix, 1 μl of each primer (the CpG WIZ Methylation Assay Kit), 2 μl of modified DNA template, and 2.6 units of Taq polymerase from the Expand High Fidelity PCR System (Boehringer Mannheim). PCR reactions were overlaid with mineral oil (Sigma Chemical Co.). The cycling parameters included an initial denaturing step at 95°C for 5 min, followed by 30 cycles of 95°C for 45 s, 60°C for 45 s, and 72°C for 1 min. Five μl of PCR reactions were analyzed by electrophoresis through a 2% agarose gel, followed by ethidium bromide staining. The size of the methylated INK4a product was 145 bp, whereas that of the unmethylated INK4a product was 154 bp.

Statistical Analysis. Follow-up data were taken from time of last clinic appointment or date of death. Median follow-up was 74 months. Of the 217 patients, 81 patients had relapsed, 54 had developed distant metastases, and 44 had died of breast cancer at the time of analysis. Deaths from unrelated causes were censored for purposes of survival analyses. All statistical calculations were performed using the SPSS Data Analysis Program (SPSS for Windows 6.1.3: SPSS UK Ltd.). The association between p16INK4a mRNA expression and other clinicopathological variables was determined by the χ² test (using Fisher’s exact (two-tailed) test). Survival outcomes were assessed using univariate Cox regression analysis, multivariate Cox proportional hazards model, and life table analysis using the Wilcoxon Gehan statistic (53). The relationship between the expression of p16INK4a mRNA and cyclin D1 or ER mRNA was determined using the nonparametric Mann-Whitney U test.

RESULTS

p16INK4a Exon 1α Expression in Breast Cancer Cell Lines. Given the complexity of the INK4a gene with two partially overlapping transcripts produced from separate promoters, a specific probe for the first exon of human p16INK4a (exon 1α) was generated by PCR to avoid cross-reaction with the first exon of p19ARF (exon 1β). A 1.6-kb transcript was clearly defined on Northern blots of RNA from a panel of breast cancer cell lines probed with full-length p16INK4a or p16INK4a exon 1α cDNA (Fig. 1A). The cell lines with known homozygous deletion [e.g., MDA-MB-231 and MCF-7 (31)] did not express p16INK4a mRNA when probed with either full-length p16INK4a or p16INK4a exon 1α cDNA (Fig. 1A). However, three additional cell lines (T-47D, MDA-MB-134, and DU-4475) lacked evidence of gene expression when the specific exon 1α PCR product was used as a probe, in contrast to the clearly defined transcript demonstrated when blotted for full-length p16INK4a, indicating that p19ARF but not p16INK4a was expressed in these cell lines. Thus, the exon 1α-specific probe for p16INK4a expression was used throughout this study.

p16INK4a Exon 1α Expression in 314 Primary Breast Cancers. Total RNA from 364 primary breast cancers was Northern blotted on 22 filters and probed sequentially with cDNAs for cyclin D1, ER, p16INK4a exon 1α, and the ribosomal protein 36B4, the last of which was used as a control for RNA loading [Fig. 1B (49, 50)]. RNAs from 50 breast cancer samples were degraded and unable to be evaluated for p16INK4a expression and were therefore excluded from the analysis. The frequency distribution of p16INK4a mRNA was unimodal and positively skewed, with the relative p16 mRNA levels ranging up to 28.26 (Fig. 1C). However, most of the samples expressed very low levels of p16INK4a with a median level of 1.76.

Relationship between p16INK4a and Cyclin D1 Expression. In cell cycle regulation, p16INK4a induces a conformational change in Cdk4 and Cdk6 that reduces their affinity for cyclin D1, thereby preventing cyclin D1-Cdk4/Cdk6 assembly and pRb phosphorylation. Thus, the relative abundance of p16INK4a and cyclin D1 may affect Cdk4/Cdk6 activity. We first examined the relationship between p16INK4a and cyclin D1 expression in 314 primary breast cancers. An inverse relationship between p16INK4a and cyclin D1 expression was clearly demonstrated (Fig. 2A). Cyclin D1 mRNA levels in the p16INK4a high expressers (using the median of p16INK4a as the cutoff) were significantly lower than those in the remainder of the population (P = 0.0002). Similarly, when the tumors were divided into halves according to their cyclin D1 levels, the relative p16INK4a mRNA expression in the half with lower cyclin D1 levels was significantly higher than that in the half with higher cyclin D1 mRNA (P = 0.0001).

Relationship between p16INK4a and ER Expression. Given that ER is a known marker of prognosis and a predictor for therapeutic responsiveness to endocrine treatment in breast cancer, the relationship between p16INK4a and ER expression was also examined. An inverse relationship between p16INK4a and ER mRNA levels was clearly shown, and this relationship was even more striking in the high expressers of p16INK4a (Fig. 2B). ER mRNA levels in the p16INK4a overexpressers were significantly lower than those in the remainder of the population (P = 0.035). p16INK4a mRNA levels in the ER-negative group were significantly higher than those in the ER-positive group (P = 0.0001). Of the 10% of tumors (i.e., 32 samples) that expressed the highest levels of p16INK4a mRNA, only six were ER positive, and their relative ER mRNA levels were all very low (<0.3 arbitrary units). When p16INK4a mRNA levels were divided into quartiles, the proportion of ER-negative samples increased from 15% in all of the lowest three quartiles to 49% in the highest quartile.

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Fig. 1. A, expression of full-length p16\textsuperscript{INK4a} and p16\textsuperscript{INK4a} exon 1α mRNA in breast cancer cell lines. A Northern blot containing 30 μg of RNA from 11 breast cancer cell lines is shown. The filter was probed sequentially with p16\textsuperscript{INK4a} exon 1α and full-length p16\textsuperscript{INK4a} cDNAs. The ER status and pRB status of these cell lines are shown (31).

B, expression of p16\textsuperscript{INK4a} mRNA in primary breast cancers. A representative Northern blot containing the control breast cancer cell lines HBL-100, MDA-MB-231, MCF-7M, and MDA-MB-134 (20, 10, 20, and 5 μg of RNA, respectively) and 20 μg of RNA from each of the 18 primary breast cancer samples is shown. The filters were probed sequentially with [α-\textsuperscript{32}P]dCTP-labeled cyclin D1, ER, p16\textsuperscript{INK4a} exon 1α, and 36B4 cDNAs. The sizes of the transcripts are indicated.

C, frequency distribution of p16\textsuperscript{INK4a} mRNA levels in 314 primary breast cancers.
INK4a Methylation in Breast Cancer Cell Lines and Primary Breast Cancers. Given that a substantial proportion of breast tumors expressed very low levels of p16INK4a mRNA and that homozygous deletions and point mutations of the INK4a gene are uncommon in breast cancers (30, 31), we investigated whether the gene was inactivated by DNA methylation. Exon 1 and 2 coding sequences of the INK4a gene are investigated whether the gene was inactivated by DNA methylation (Fig. 3A). As expected, no PCR product was demonstrated in 10 of 18 breast cancer cell lines with homozygous deletion of INK4a (ZR 75-1). When the relative p16INK4a mRNA levels were divided into tertiles, 57 tumors were examined for INK4a methylation status in the first tertile (i.e., lowest expression), 33 were examined for INK4a methylation status in the second tertile, and 30 were examined for INK4a methylation status in the third tertile. Of the 24 methylated samples, 18 (32%) were in the lowest tertile of p16INK4a expression, 5 (15%) were in the second tertile of p16INK4a expression, and 1 (3%) was in the highest tertile of p16INK4a expression (Fig. 3C).

**Relationship between p16INK4a mRNA and Prognosis in a Subset of 217 Patients.** Of the 314 patients, 217 had full clinical follow-up data to allow survival analysis. The p16INK4a levels were corrected for RNA loading with the non-estrogen-regulated ribosomal protein 36B4. For analyses of the relationship between p16INK4a mRNA expression and clinicopathological parameters or survival outcome, patients were divided into two equal groups using the median p16INK4a level as the cutoff point.

The relationship between p16INK4a mRNA expression and various clinicopathological parameters is summarized in Table 2. High p16INK4a mRNA expression was associated with increased risk of death from breast cancer. In contrast, no relationships between high p16INK4a mRNA and patient age, menopausal status, and tumor size were evident.

Life-table analysis revealed that high p16INK4a mRNA expression was associated with increased risk of relapse in the whole population of 217 patients (P = 0.006; Fig. 4A). Although the overall survival statistics failed to reach significance using the median expression as the cutoff, there is an apparent divergence of the cumulative proportion overall survival curves with a trend for worse prognosis in patients with high p16INK4a mRNA levels (P = 0.082; Fig. 4B). The association with increased risk of death became statistically significant when the analysis was performed using p16 expression as a continuous variable (P = 0.0037). The Cox multivariate analyses of several pathological features using relapse-free survival and overall survival as end points showed that axillary LN involvement >3 (P = 0.0330) and tumor size >2 cm (P = 0.0289) were associated with increased risk of relapse, whereas axillary LN >3 (P = 0.0433) and high tumor grade (P = 0.0096) were associated with increased risk of death from breast cancer. In contrast, p16 overexpression was not an independent predictor of early relapse or death (P = 0.6558 and 0.0766, respectively). This is probably not surprising given the relationship between p16 overexpression and LN status, which is the single most important independent predictor of outcome.

Cyclin D1 mRNA overexpression is associated with worse prognosis in patients with ER-positive but not ER-negative breast cancers (48). Moreover, patients with ER-negative disease generally have a less favorable outcome, and given the tight inverse relationship between p16INK4a mRNA expression and ER, the reduced disease-free survival in patients with high p16INK4a mRNA expression may be accounted for by its asso-
Association with ER negativity. Thus, survival analyses within the ER subgroups were performed. The association between high p16INK4a mRNA expression and early relapse was upheld within the ER-positive subgroup \((P = 0.04;\) Fig. 5A). Unfortunately, the small sample size within the ER-negative group \((n = 54)\) did not provide enough events to allow meaningful statistical analysis. There was no association between p16INK4a mRNA expression and disease-free survival within the ER-negative subgroup.

Given that axillary LN status is the single most important prognostic indicator in breast cancer and that high p16INK4a mRNA expression is associated with increased nodal involvement \((\geq 4\) LNs), survival analyses within node-negative and node-positive subgroups were performed. In common with the whole population survival analyses \((n = 217)\), the association between high p16INK4a expression and early relapse was maintained in the larger node-positive subgroup \((n = 154;\) \(P = 0.0382;\) Fig. 5B). However, this association was lost in the much smaller node-negative subgroup. 

Fig. 3 Methylation status of INK4a in (A) breast cancer cell lines and (B) primary breast cancers. Representative gel analyses of methylation-specific PCR reactions on DNA from six breast cancer cell lines and PBLs or six breast tumor samples are shown. A PCR fragment of 145 or 154 bp in size was evident if INK4a was originally methylated or unmethylated in the cell line or breast cancer sample, respectively. C, relationship between INK4a methylation status and INK4a gene expression. Data are presented as the percentage of primary breast cancer samples with methylated and unmethylated INK4a in each tertile of relative p16INK4a mRNA expression.
smaller node-negative subgroup (n = 33). High p16INK4a mRNA expression had no impact on overall survival in either nodal subgroup.

Because high cyclin D1 (48) and high p16INK4a mRNA expression were each a marker of worse prognosis in this series of patients, we examined the potential prognostic significance of the combination of these two parameters. Although there was clearly an inverse relationship between p16INK4a and cyclin D1, there was a significant proportion of tumors exhibiting moderate overexpression of both parameters when the median was used as the cutoff. Patients with tumors expressing high cyclin D1/high p16INK4a mRNA levels had increased risk of early relapse as compared with the low cyclin D1/low p16INK4a mRNA expression (Fig. 6A; P = 0.0164). Similarly, the group with low cyclin D1 levels but high p16INK4a mRNA levels had an increased risk of early relapse as compared with the group with concurrently low cyclin D1 and p16INK4a mRNA expression (P = 0.0340). The association between high expression of both cyclin D1/p16INK4a mRNA and early relapse as compared with low expression of both cyclin D1/p16INK4a mRNA reached greater statistical significance within the ER-positive subgroup (Fig. 6B; P = 0.0054). There was no survival difference between patients with high cyclin D1/low p16INK4a and low cyclin D1/high p16INK4a mRNA levels in the whole population or within ER subgroups.

These data demonstrate that independent and concurrent overexpression of cyclin D1 and p16INK4a mRNAs were markers of poor prognosis in this series of breast cancers, but little is known of the prognostic value of p16INK4a inactivation. Consequently, survival analyses were carried out in relation to INK4a methylation status. Of the subset of 120 breast cancers for which methylation data were available, 97 patients had adequate follow-up data to allow survival analyses. No association was demonstrated between INK4a methylation status and the overall or disease-free survival in this group of patients.

**DISCUSSION**

Cyclin D1 and Cdk4 accelerate, whereas p16INK4a and pRb inhibit, cell cycle progression at the late G1 phase of the cell cycle. The loss of functional p16INK4a or pRb has been identified in a variety of human cancers but has not been well studied in breast cancer. This study provides extensive clinical data on p16INK4a expression in breast cancer. For the first time, a tight inverse relationship between p16INK4a and ER mRNA expression was demonstrated. Moreover, the findings also indicated that both p16INK4a inactivation by hypermethylation and p16INK4a overexpression occur frequently in primary breast cancer, and overexpression of p16INK4a mRNA is a marker of poor prognosis.

Growth-suppressive effects of p16INK4a generally require functional pRb (54, 55). The observation of high levels of p16INK4a expression in pRb-negative cells, which is likely due to loss of a feedback loop regulated by pRb (13–16, 56), and the fact that pRb expression is transcriptionally repressed by ectopic expression of p16INK4a (18) suggest that high p16INK4a levels may be a marker of pRb inactivation or low pRb expression. On the other hand, the loss of p16INK4a would likely lead to increased cyclin D1/Cdk4 activity. One study indicated that cyclin D1 and p16INK4a alterations can cooperate to deregulate G1 control, resulting in multistep tumorigenesis (3). However, the inverse relationship between p16INK4a and cyclin D1 in this series of breast cancers supports the hypothesis that there is no selective advantage for aberration of more than one of these genes. Given that overexpression of cyclin D1, p16INK4a inactivation, and pRb inactivation are frequently mutually exclusive,

### Table 1 INK4a methylation status in normal and breast cancer cell lines

<table>
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<tr>
<th>Cell lines</th>
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<th>Methylated reaction</th>
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<td>184</td>
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<td>Unmethylated</td>
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<td>PBLs</td>
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### Table 2 The relationship between p16INK4a mRNA expression and clinicopathological features in 217 breast cancer patients

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<td>Postmenopausal (n = 131)</td>
<td>69</td>
<td>62</td>
<td>0.412</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td></td>
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<tr>
<td>Not known (n = 10)</td>
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<td>3</td>
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<tr>
<td>&lt;2 (n = 88)</td>
<td>48</td>
<td>40</td>
<td></td>
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<tr>
<td>≥2 (n = 119)</td>
<td>54</td>
<td>65</td>
<td>0.192</td>
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<tr>
<td>Tumor grade</td>
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<tr>
<td>Not known (n = 12)</td>
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<tr>
<td>1 (n = 80)</td>
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<td>33</td>
<td></td>
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<tr>
<td>2 (n = 97)</td>
<td>44</td>
<td>53</td>
<td></td>
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<tr>
<td>3 (n = 28)</td>
<td>7</td>
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<td>0.006*</td>
</tr>
<tr>
<td>LN status</td>
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<tr>
<td>Not known (n = 29)</td>
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<tr>
<td>Negative (n = 33)</td>
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<td>13</td>
<td></td>
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<tr>
<td>Positive 1–3 nodes (n = 53)</td>
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<td>21</td>
<td></td>
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<tr>
<td>Positive ≥4 nodes (n = 102)</td>
<td>41</td>
<td>61</td>
<td>0.021*</td>
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<td>Not negative (n = 54)</td>
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<td>42</td>
<td></td>
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<tr>
<td>Positive (n = 163)</td>
<td>97</td>
<td>66</td>
<td>0.0001*</td>
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* Significant (P < 0.05).
In this study, together with the previous observation of cyclin D1 mRNA overexpression in 45% of breast tumors from a similar series (57), indicated an overall rate of perturbation of the Rb pathway of at least 80% in primary breast cancer (i.e., 45% cyclin D1 overexpression, 20% INK4a hypermethylation, and 16% p16 INK4a overexpression).

The evidence for cyclin D1 induction by estrogen (58–60) and the demonstration of a tight correlation between cyclin D1 and ER gene expression in breast cancers (49, 61) may account, at least in part, for the inverse relationship between p16INK4a and ER mRNA expression reported in this study. ER function can up-regulate cyclin D1 expression, which, in turn, increases phosphorylation of functional pRb, and this may then negatively modulate INK4a transcription. However, this inverse relationship appeared to be even tighter in patients with high p16 INK4a expression, considering that only six tumors in the top 10% of p16INK4a levels were ER positive, and all had very low levels of ER. This is unlikely to be fully explained by the association between low cyclin D1 expression and ER negativity. One study (62) indicated that estrogen decreases the expression of pRb at the level of protein and mRNA by a posttranscriptional mechanism. However, the relationship between pRb and ER in breast cancer has been controversial (16, 63). The inverse relationship between p16INK4a and ER status in this study may suggest that high p16INK4a levels could reduce the requirement for estrogen for proliferation of breast cancer cells. Thus, further investigation will be required to define the precise mechanisms responsible for the relationship between ER and p16INK4a or pRb gene expression. Furthermore, this tight inverse relationship between p16INK4a and ER may indicate that high expression of p16INK4a may be associated with a lack of hormone responsiveness in breast cancer.

De novo methylation of CpG islands within the gene promoter of tumor suppressor genes is an alternative pathway of transcriptional inactivation providing a selective growth advantage to tumor cells (64). The methylation status of INK4a in a few breast cancer cell lines has been determined previously (65) using Southern analysis to detect differential restriction enzyme cleavage from non-methylation-sensitive and methylation-sensitive restriction enzymes. Methylation-specific PCR, however, eliminates the false positive results inherent in Southern analysis. In this study, the MCF-7, MDA-MB-231, Hs-578T, and BT-20 cell lines were negative in PCR reactions using both methylated- and unmethylated-specific primers, indicating that INK4a was homozygously deleted, a result consistent with previous findings (31). As expected, the three breast cancer cell lines T-47D (65), MDA-MB-134 (66), and DU-4475 with methylated INK4a had undetectable p16INK4a mRNA expression (Fig. 1). Similarly, all breast cancer cell lines with high p16INK4a expression (MDA-MB-157, MDA-MB-436, BT-549, MDA-MB-468, and HBL-100; Fig. 1) had unmethylated INK4a (Table 1).

Fig. 4 Life-table analysis with cumulative proportion of (A) disease-free survival and (B) overall survival for the total population of patients \( (n = 217) \) in relation to low (○) or high (●) p16INK4a mRNA levels.

Fig. 5 Relationship between p16INK4a mRNA expression and disease-free survival in patients with (A) ER-positive and (B) axillary LN-positive breast cancers. Low p16INK4a mRNA levels, ○; high p16INK4a mRNA levels, ●.
Unlike breast cancer cell lines, most of the breast tumor samples, as shown in Fig. 3B, displayed products from both methylated and unmethylated PCR reactions. This is most likely due to the inevitable admixture of DNA extracted from both cancer cells and the surrounding normal stromal cells. Thus, if a PCR fragment was evident in the methylated reaction, irrespective of the unmethylated reaction, INK4a was regarded as methylated in this tumor sample. The marked reduction in the number of tumor samples exhibiting INK4a methylation with ascending tertiles of p16INK4a mRNA expression was expected. However, the discovery of five INK4a methylated tumors within the middle tertile of p16INK4a expression and one INK4a methylated tumor in the highest tertile may indicate that Northern analysis is limited in detecting complete loss of tumor p16INK4a expression, given the admixture of normal cells. Moreover, methylation of p16 exon 1 was assessed in this study, rather than methylation of its upstream promoter region, which might correlate better with expression. In the current series of breast cancers, ~20% demonstrated INK4a methylation, which is consistent with a previously published smaller study (65). Thus, unlike the low frequency of gene deletion or mutation, INK4a methylation occurs reasonably frequently, and to date, it is by far the most commonly documented mechanism of inactivation of the INK4a gene in primary breast cancer. However, the survival data failed to show even a trend or a relationship between INK4a methylation status and outcome. Additional INK4a methylation assays in a larger series of patients will be necessary to fully investigate any prognostic significance of INK4a methylation.

A recently published study (16) indicated that strong immunohistochemical staining of p16INK4a was associated with increased risk of death in 191 breast cancer patients. INK4a is a tumor suppressor gene, and tumorigenesis is expected as a consequence of inactivation of the gene, but not from gene overexpression. However, high p16INK4a expression may be indicative of inactivation of pRb (13, 16, 56). The relationship between pRb inactivation and clinical outcome in breast cancer has been controversial. One study indicated an association between abnormal pRb expression and an aggressive phenotype (67), whereas another reported an association between RB gene alterations and favorable prognostic factors (63) in breast cancer. A number of groups failed to demonstrate a relationship between pRb aberration and patient outcome (16, 63, 67). On the other hand, overexpression of p16INK4a may be independent of pRb mutation, as indicated in recent studies in ovarian (68) and prostate cancer. Nevertheless, although Northern blot analysis was not sensitive enough to detect all p16INK4a inactivation and may have underestimated the number of samples with real overexpression of p16INK4a, the current study supports earlier evidence (16) that p16INK4a overexpression is an indicator of poor prognosis in primary breast cancer.

In conclusion, loss of p16INK4a expression, overexpression of cyclin D1, and low expression or function may have similar effects on G1 progression and may represent a common pathway in tumorigenesis. Our findings suggest that both overexpression of p16INK4a and de novo INK4a methylation occur frequently in primary breast cancers. Furthermore, high p16INK4a mRNA expression is associated with aggressive clinicopathological features in primary breast cancer. The demonstration of a significant negative correlation between the expression of the INK4a and ER genes raises issues regarding their functional interrelationships and, consequently, their roles as potential therapeutic response parameters.

ACKNOWLEDGMENTS

We thank Matthew Mitchell for invaluable assistance with the statistical analysis. We are also indebted to Ann L. Cornish and Richard A. McClelland for help with preparation of the tumor RNA samples.

REFERENCES


Clinical Cancer Research

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