Somatic Mutations and Altered Expression of the Candidate Tumor Suppressors CSNK1ε, DLG1, and EDD/hHYD in Mammary Ductal Carcinoma

Tannin J. Fuja, Fritz Lin, Kathryn E. Osann, and Peter J. Bryant

1Developmental Biology Center and 2Department of Medicine, University of California–Irvine, Irvine, California, and 3Department of Pathology, University of California Irvine Medical Center, Orange, California

ABSTRACT

We report somatic mutations in three genes (CSNK1ε, encoding the Ser/Thr kinase casein kinase 1 ε; DLG1, encoding a membrane-associated putative scaffolding protein; and EDD/hHYD, encoding a progesterin-induced putative ubiquitin-protein ligase) in mammary ductal carcinoma. These genes were suspected of playing a role in cancer because loss-of-function mutations in their Drosophila homologues cause excess tissue growth. Using DNA from 82 laser-microdissected tumor samples, followed by microsatellite analysis, denaturing HPLC and direct sequencing, we found multiple somatic point mutations in all three genes, and these mutations showed significant association with loss of heterozygosity of closely linked polymorphic microsatellite markers. For CSNK1ε and DLG1, most of the mutations affected highly conserved residues, some were found repetitively in different patients, and no synonymous mutations were found, indicating that the observed mutations were selected in tumors and may be functionally significant. Immunohistochemical reactivity of each protein was reduced in poorly differentiated tumors, and there was a positive association between altered protein reactivity, loss of heterozygosity, and somatic mutations. There was a statistically significant association of hDlg staining with p53 and Ki67 reactivity, whereas CSK1ε and EDD/hHYD staining levels were associated with progesterone receptor status. The results provide strong indications for a role of all three genes in mammary ductal carcinoma. They also justify additional studies of the functional significance of the changes, as well as a search for additional changes in these and other genes identified from studies on model systems.

INTRODUCTION

Mammary carcinoma is one of the most common neoplasms in women (1), and it is the second leading cause of cancer-related deaths in women of the Western world (2). Despite significant improvements in earlier detection and diagnosis of this cancer leading to more effective treatment modalities, approximately one-quarter of breast cancer patients still die of their disease (2). Understanding the molecular pathology of mammary carcinoma is critical for the development of novel, molecular-based therapies (3, 4). Progress has been made in identifying tumor suppressor genes affected in hereditary forms of breast cancer such as BRCA1 and BRCA2 (5). However, ~95% of breast cancers are sporadic rather than hereditary (6), and very little progress has been made in identifying the molecular lesions responsible for these cases. Here, we show that at least some of the somatic genetic changes occurring in sporadic breast cancer can be identified using a candidate gene approach.

Mammary carcinoma develops through defined clinical and pathological stages, starting with atypical epithelial hyperplasia, progressing to carcinoma in situ, then invasive carcinomas, and culminating in metastatic disease (7). The most common type of breast cancer is invasive ductal carcinoma, which develops from ductal carcinoma in situ (DCIS) and spreads through the duct walls to invade the breast tissue (8). Although the morphological manifestations are well characterized, the molecular events involved in this progress have not been identified (9).

Drosophila has been used as a model organism to identify genes involved in the regulation of cell proliferation and tumorogenesis (10). Genetic studies have led to the identification of tumor suppressor genes (TSGs) in which recessive mutations lead to neoplastic or hyperplastic overgrowth of larval imaginal discs (specialized epithelial cells) and other tissues, mainly in the larva (11, 12). Using tissue microarrays, we confirmed that the human homologues of three of these TSGs (CSNK1ε, DLG1, and EDD/hHYD) are expressed in normal human breast tissue. Each of them has functional and sequence characteristics, suggesting that it may play a role in regulating mammary cell growth:

CSNK1ε. This gene encodes casein kinase 1-ε (CK1ε), a serine-threonine kinase whose overexpression mimics Wnt signaling by stabilizing β-catenin, thereby increasing expression of β-catenin–dependent genes. CK1ε is homologous to the product of the Drosophila TSG dco (discs overgrown) in which some allelic combinations result in overgrowth phenotypes (13, 14). CK1ε is found in the cytoplasm as a monomer and can phosphorylate a variety of proteins, including itself (15, 16).

DLG1. DLG1 encodes hDlg, which is a member of the protein family named membrane-associated GUAnylate kinase homologues (17, 18). It is a homologue of the Drosophila TSG dlg (discs-large) in which loss-of-function mutations result in overgrowth of the imaginal discs and larval brain. Membrane-associated GUAnylate kinase homologues are usually localized at the membrane cytoskeleton and contain several domains, suggesting a role in intracellular signal transduction (19). Immunohistochemical localization experiments show that hDlg is localized along the basolateral surfaces of epithelial cells in the intestine and at the plasma membrane at sites of cell-cell contact in cultured colon and mammary epithelial cells (20). hDlg expression is down-regulated in both cervical (21) and gastric (22) cancer, and loss of the protein at cell contact sites may be an important step in development of epithelial cancers (17, 21, 23, 24). The PDZ2 domain of hDlg binds TOPK/PBK, a novel mitotic kinase, and hDlg also interacts with other tumor suppressors, notably APC (25) and Pten (26), both of which are involved in human cancers. The hDlg protein is highly enriched at the membrane of normal epithelial cells, but it shows a higher level of cytoplasmic accumulation in high-grade cervical neoplasia (21). It is a primary target of the E6 oncprotein produced by high-risk strains of papillomavirus (27).

EDD/hHYD/DD5. EDD encodes a progesterin-induced protein belonging to the HECT family (28). The HECT family proteins function as E3 ubiquitin-protein ligases, targeting specific proteins for ubiquitin-mediated proteolysis (29, 30). EDD is a homologue of the product of Drosophila hyd (hyperplastic discs) in which loss-of-function mutations cause imaginal disk overgrowth (31). It interacts with importin α-5 through basic nuclear localization signals and binds to the progestosterone receptor to potentiate progesterin-mediated gene activation (28). Furthermore, EDD interacts with the DNA topoi-
somerase II-β-binding protein I through its BRCT [BRCA1 COOH terminus domain (32)]. These domains are involved in cell cycle arrest in response to activation of the DNA damage checkpoint.

To explore the potential role of these three candidate TSGs in mammmary ductal carcinoma, we obtained DNA from tumor samples by laser capture microdissection (LCM). We tested for loss of heterozygosity (LOH) of polymorphic markers flanking each gene and PCR-amplified genomic fragments encoding critical regions of each protein. These fragments were used for mutation detection using denaturing high-performance liquid chromatography (dHPLC) and direct sequencing. For each gene, we also characterized expression and localization of the protein product in paraffin-embedded sections of mammary ductal carcinomas using immunoperoxidase and immunofluorescence histochemistry. The results provide strong indications for a role of all three genes in mammary ductal carcinoma.

MATERIALS AND METHODS

Tissue Samples. Eighty-two cases of formalin-fixed, paraffin-embedded, archived mammary ductal carcinoma and case/patient-specific paired samples of normal tissue were collected from the University of California–Irvine, Department of Pathology Core Facility, under the direction and approval of the University of California Human Subjects Institutional Review Board. In cases showing significant intratumor heterogeneity (e.g., regions of clear DCIS isolated from regions of invasive carcinomas), the two disease areas were treated independently, so the total case number described in the results is 85. Samples were accompanied by pathology reports that had been stripped of any patient identifiers or links. These reports were held and corroborated by Dr. Fritz Lin, a certified pathologist and chief of surgical pathology at University of California Irvine Medical Center.

Twenty serial 5-μm thick sections were taken from each tissue block. To confirm the maintenance of the diagnosis of each specimen throughout the 20 sections, standard Meyer’s H&E Y stains were performed at even intervals throughout the entire series. These control H&E stains were reviewed by Dr. Lin to ensure consistency of diagnosis. The Scarff-Bloom-Richardson (SBR) scoring system (33) was used to classify morphological grades of ductal carcinoma.

LCM. Homogeneous regions of cancer or normal tissue from each case were obtained using the PixCell IIe Laser Capture Microdissection System (Acturus Engineering, Inc., Mountain View, CA; Ref. 34). Each region captured was confirmed as cancer or normal by pathologist review. Using recommended settings (34), 2000 laser hits were taken from each case. To increase the chance of finding genetic variation, cells were captured from adjacent locations within the same section as well as different regions of the tumor from each patient, and the samples were mixed.

DNA Extraction. DNA was extracted in 50 μl of freshly made DNA digestion buffer [0.08% proteinase K, 15 mM Tris- HCl (pH 8.0), 1 mM EDTA, 0.5% Tween 20], which was preheated to 42°C and incubated overnight. The contents of the tube were collected by centrifugation and then heated to 95°C for 8 min. To remove eosin, the DNA was purified using a modified Qiagen DNeasy protocol (Qiagen, Valencia, CA) with serial ethanol washes.

Genomic Mapping and PCR Amplification. We analyzed heterozygosity for the following polymorphic microsatellite markers flanking each locus on the Centromeric (C) and telomeric (T) sides: CSK1ε: d22s1156 (305 Kb C), d22s272 (399 Kb T), and d22s423 (1659 Kb T); and d3s1272 and d3s1265—52.7°C. The optimal annealing temperature of the skeletal α-skeletal actin control was 60°C. After 3 min of incubation at 94°C, amplification was carried out by 35 cycles of: 94°C (30 s), T° (30 s), and elongation 72°C (1 min). These cycles were followed by a final elongation step at 72°C for 7 min.

Compatibly labeled microsatellite fragments were pooled and scored with the ABI PRISM 3100 Genetic Analyzer. Using the ABI Genescan and ABI Genotyper software, cases were scored as informative for a polymorphic marker if the DNA was effectively amplified and the normal DNA was heterozygous for that marker. LOH was characterized according to the equation Na/Nb = (Ca/Cb), where Na and Nb were the peak height or peak area of the two predominant alleles within the specific microsatellite size range of the normal DNA, and Ca and Cb were the peak height or peak area of the corresponding alleles in the cancer DNA. When v ≤ 0.5 or ≥ 2, the case was scored as LOH for that marker (4, 39). PCR and fragment analysis were repeated three times for each case. When the sample did not show heterozygosity in the normal tissue or did not show conservation of at least one allele in the cancer as compared with normal, it was scored as noninformative and not included in the LOH analysis.

dHPLC. Ion-pair reversed-phase dHPLC identifies mutations by detecting heteroduplex formation between mismatched nucleotides in PCR-amplified DNA (40). Using the WAVE Nucleic Acid Fragment Analysis System (Transgenomics, Inc., Omaha, NE), we analyzed selected regions of each candidate TSG for the presence of mutations (41, 42). Amplons encoding the following functional domains were selected for dHPLC analysis: the kinase domain of CKIε; the PDZ2 domain of hDlg; and the NH-terminal region of the EDD HECT domain. A section of the 5′-untranslated region of ACT1 was used as a control. After identifying the melt domains of each fragment, we tested the samples for heteroduplex formation at the two best temperatures.

Direct Sequencing. DNA from LCM samples was sequenced using ABI Big Dye Terminator Ready v.3.1 cycle sequencing kits (PE Applied Biosystems), after Shrimp Alkaline Phosphatase/Exonuclease I 1-h digestion at 37°C, followed by heat inactivation at 70°C for 15 min. (Promega Corp., Madison, WI). Bi-directional cycle sequencing was performed to confirm each sequence. The mixture of 60 ng of PCR-amplified SAP/Exo I-digested DNA, 2.3 pmol of forward or reverse primer, 1× buffer [1 mM Tris (pH 9.0) and 25 mM MgCl2] supplemented with PCRx Enhancer (Invitrogen-Life Technologies, Inc.) was held at 95°C for 5 min, followed by 90 cycles of 95°C (30 s), 50°C (10 s), and 60°C (4 min). The resulting material was precipitated in 75% isopropanol and air-dried. The ABI recommends 1 μl of the dried samples were run on a 3130 ABI DNA Analyzer, and sequence data were evaluated using Chromas v1.45 (Technelysium).

Immunohistochemistry. To examine protein expression in situ, paraffin-embedded sections were analyzed by indirect immunolabeling using both peroxidase and fluorescence-based histochemical methods (43). Two sections from each case were stained using the same immunoperoxidase protocol, and these sections were processed by two different technicians to monitor for variability resulting from staining procedure. A third section was stained for immunofluorescence to ensure staining patterns were not biased by the use of a single staining method. Commerically available, affinity-purified goat polyclonal anti-CKIε (C-20) raised against a COOH-terminal peptide of CKIε (Santa Cruz Biotechnology, Santa Cruz, CA) was used at 10 μg/ml. Mouse IgG1 monoclonal anti-hDlg raised against amino acid 1-229 of hDlg was used at 7 μg/ml (Santa Cruz Biotechnology). Affinity-purified goat polyclonal anti-EDD raised against a peptide mapping near the COOH terminus of EDD was used at 5 μg/ml (Santa Cruz Biotechnology). Data showing the specificity of these antibodies is available from the company’s web site (www.scbt.com). For fluorescently labeled sections, Alexa Fluor 488 and 568 goat antimouse IgG (H+L) and Alexa Fluor 488 and 568 donkey antigoat (Molecular Probes, Inc., Eugene, OR) were used at 5 μg/ml ToProIII (Molecular Probes, Inc.,) was used to fluorescently label DNA.

Immunofluorescent-labeled sections were viewed using an MRC 1024 Bio-Rad/Nikon Diaphot 200 laser-scanning confocal microscope and analyzed
using LaserSharp image analysis software (Bio-Rad Microscience Division, Cambridge, MA). Immunoperoxidase-labeled sections were observed using an Olympus BH-2 light microscope equipped with a Sony DYC-960-MD3 charge-coupled device (CCD) camera, and images from this system were collected and analyzed using Pax-It Image software.

**Scoring of Protein Reactivity.** For each case, the intensity of CK1ε, hDlg, and EDD staining in mammary ductal tumors was compared with case-matched (from the same patient) normal ductal tissue. Each case was independently scored three times in a single-blind manner to reduce scoring bias. In cases where different regions of a section gave scores differing by greater than two scoring units (according to the scale below), each region was reported independently. Otherwise, the mean stain score was used. A comparative scale from 1 to 5 was used to score staining intensity: 1, much less staining in the cancer tissue than in the normal tissue; 2, moderately less staining in the cancer tissue than in the normal tissue; 3, same intensity of stain in the cancer as in the normal; 4, moderately more staining in the cancer than in the normal; and 5, much more staining in the cancer tissue than in the normal tissue.

This relative score reflects the difference between normal tissue and tumor tissue. A mean score of 3.0 indicates no difference between normal and tumor tissue overall, whereas a mean score that exceeds 3.0 by two or more SEs indicates significant overexpression in tumor compared with normal tissue.

Tissue sections were also stained for the following standard clinical indicators: estrogen receptor (ER), progesterone receptor (PR), p53, Ki67, and ErbB2 (HER-2/Neu). These were detected using only the immunoperoxidase labeling method. These indicators were scored positive or negative using standard clinical pathology guidelines and parameters.

**Statistical Analysis.** Data were analyzed using standard methods appropriate for continuous and categorical data, including t tests, ANOVA, and χ² tests. The average staining score for each of the three TSGs was treated as a continuous normally distributed variable. Differences between invasive cancer samples and DCIS were evaluated using t tests and ANOVA. For comparisons between three or more groups (ANOVA), pairwise t tests were conducted with Bonferroni adjustment for multiple comparisons. Associations between level of protein staining for TSGs and standard clinical indicators, including ER, PR, p53, Ki67, and ErbB2 (HER-2/Neu), were investigated using methods appropriate for categorical data. The Cochran χ² test for linear trend was used to test for an increasing or decreasing trend in the proportion of subjects for which average staining levels were <3 (less than normal tissue), equal to 3 (the same as normal tissue), or >3 (greater than normal tissue).

The reliability of the mean of n raters has been estimated as the variance due to the mean of the errors of measurement. Reliability can be estimated as (F) variance due to true scores divided by the sum of the variance due to true scores and the error variance. The reliability of the mean of n raters has been estimated as (F) variance due to the mean of the errors of measurement. Reliability can be estimated as the variance due to true scores divided by the sum of the variance due to true scores and the error variance.

**RESULTS**

**Somatic Mutations.** dHPLC was used to screen for somatic mutations in regions of each gene that encode protein domains with known functions. Case-specific DNA extracted from normal and cancer tissue was PCR-amplified using gene-specific genomic primers. The DNA encoding the following protein regions was amplified: the NH₂-terminal portion of the Ser/Thr Kinase domain of CSNK1ε; the NH₂-terminal region of the second PDZ domain of hDlg; and the NH₂-terminal portion of the HECT domain of EDD/hHYD. After PCR amplification and agarose gel band confirmation, the samples were run on the WAVE Transgenomic dHPLC system. To confirm the mutant sequences, dHPLC was performed three times on each sample. Eight cases showed mutations in CSNK1ε, 9 showed mutations in DLG1, and 9 showed mutations in EDD/hHYD. We have not yet tested the effects of any of these mutations on protein function.

**CSNK1ε.** Direct sequence analysis of the mutations detected by dHPLC revealed 19 nonsynonymous mutations and no synonymous mutations in CSNK1ε (Fig. 1A). The following missense mutations were identified: I29T (acr→aac); A36G (gcc→ggc); L39Q (ctg→cag); I348V (atc→tgt); and N78T (aac→acc); and S101R (aac→agg); detected twice; L49Q (ctg→cag); detected three times; 328 (338 348 358 359 360 373)

**DLG1.** We found 13 nonsynonymous mutations and no synonymous mutations in DLG1 (Fig. 1B). The following missense mutations were identified: G338R (gga→cga); found twice; L39Q (ctg→cag); detected three times; Y349S (tat→ttc); K352R (aaa→agga); K352E (aaa→geaa); G356A (gaa→gaa); H360Q (cgc→cga); found twice; and H360N (cgc→cag). Six of the seven residues altered in these cases are conserved from Drosophila to human. There was a single nonsense mutation Q340* (cag→tag).

**EDD/hHYD.** This was the only gene tested that showed silent (synonymous) mutations. We found four synonymous and six nonsynonymous mutations in this gene (Fig. 1C). The following missense mutations were detected: A2411G (gca→gga); M2419V (atg→gtg); detected twice; E2422K (gaa→gag); Q2427E (cag→cag); and A2433G (gca→gaa). All of the missense mutations predicted conservative amino acid substitutions. Three of the five residues altered in these cases are conserved from Drosophila to human. A 185-bp region of the 3’-untranslated region of the human skeletal α-actin gene was amplified as an internal control for dHPLC and

**CSNK1ε (N-terminal Ser/Thr Kinase Domain)**

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**DLG1 (N-terminal PDZ2 Domain)**

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**EDD/hHYD (N-terminal HECT Domain)**

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Fig. 1. Summary of mutations. Missense and nonsense mutations found in CSNK1ε (A), DLG1 (B), and EDD/hHYD (C). Bold: nonconservative mutations; underlined: ATP binding sites in CK1ε; star: mutation to stop codon. Scoring of nonsynonymous mutations as in Ref. 58.
direct sequencing. This analysis was done on all 13 cases that showed mutations in one or more of the three candidate genes analyzed. No heteroduplex patterns resulted from dHPLC analysis of this fragment. These DNA fragments were sequenced and found to have no detectable mutations, indicating that the mutations we detected in the three TSGs were not the result of widespread genomic instability.

**LOH.** LOH was observed at the following loci at the stated frequency: d22s1156 (15 of 39; 38.5%); d22s272 (9 of 42; 21.4%); d22s423 (5 of 38; 13.2%); d8s1789 (7 of 35; 20.0%); d8s521 (4 of 28; 14.3%); d8s1834 (3 of 39; 7.6%); d3s1265 (5 of 41; 12.2%); and d3s1272 (9 of 32; 28.1%).

**Association between Mutations and LOH.** We compared the mutation detection by dHPLC with LOH detected by fluorescent microsatellite fragment analysis. We found that those microsatellite markers that mapped more closely to the given candidate TSG locus showed statistically significant association between LOH and mutation in the gene. LOH of the **CSNK1e** microsatellite marker d22s1156 was significantly associated with **CSNK1e** mutations \( (P = 0.0062, \chi^2 \text{ test}) \); LOH of the telomeric **DLG1** microsatellite marker d3s1272 was significantly associated with **DLG1** mutations \( (P = 0.0027, \chi^2 \text{ test}) \); and LOH of the centromeric microsatellite marker of **EDD**, d8s521, was significantly associated with **EDD/hHYD** mutations \( (P = 0.033, \chi^2 \text{ test}) \).

**Immunohistochemical Reactivity of CK1e.** CK1e gave a fairly uniform cytoplasmic staining pattern (Figs. 2 and 3, A–H), although in some cases the staining was slightly more intense near the cell membrane. In the 85 cases evaluated for CK1e reactivity, this protein showed increased reactivity (mean 3.98; SE = 0.22; \( n = 17 \)) in DCIS, whereas invasive carcinoma (mean 3.04; SE = 0.13; \( n = 68 \)) showed little difference from normal mammary tissue. The difference between CK1e staining in DCIS and invasive carcinoma was statistically significant \( (P = 0.00007, t \text{ test}) \). Therefore, CK1e appears to be overexpressed in DCIS. CK1e staining was also found to vary according to SBR score in a statistically significant manner \( (P = 0.006, f \text{ test}) \). Well-differentiated ductal carcinoma gave a mean staining score of 3.93 (SE = 0.29), with moderately differentiated ductal carcinoma giving a mean staining score of 2.95 (SE = 0.16), and poorly differentiated mammary carcinoma giving a score of 2.76 (SE = 0.22). Thus, CK1e reactivity decreased in a statistically significant manner with increasing SBR Score. The reliability of the mean score from the three observations was defined as the variance due to true scores divided by the sum of the variance due to true scores and the variance.
due to the mean of the errors of the three scores. The three staining scores showed good agreement with a reliability estimate of 0.889.

**Immunohistochemical Reactivity of hDlg.** In DCIS cases, hDlg was up-regulated with a mean staining score of 3.91 (SE = 0.14, n = 17), whereas in invasive cases, it was down-regulated, giving a score of 2.87 (SE = 0.11, n = 66). The difference in staining intensities of hDlg in DCIS versus invasive carcinoma was statistically significant (P < 0.000005). Additionally, hDlg expression was reduced with increasing SBR score. Well-differentiated mammary ductal carcinoma gave a mean score of 3.26 (SE = 0.25), whereas moderately differentiated ductal carcinoma gave a mean score of 2.91 (SE = 0.15), and poorly differentiated mammary ductal carcinoma gave a mean staining score of 2.58 (SE = 0.21; P = 0.119, t test).

Also, the membrane localization of hDlg was evaluated semiquantitatively (Figs. 3, I–P and 4). It was localized to the membrane in normal mammary epithelial ducts, but membrane localization was lost with increasing SBR score, and expression was intensely cytoplasmic in DCIS cases. The reliability estimate for hDlg scoring was 0.842, showing good statistical agreement (44).

**Immunohistochemical Reactivity of EDD/hHYD.** EDD/hHYD showed nuclear localization in normal mammary tissue as previously described in T47 mammary cell lines; however, in cancer sections, staining was also reproducibly observed in the perinuclear cytoplasm (Figs. 3, Q–T and 5). EDD/hHYD showed slightly increased staining intensity in DCIS (mean 3.41; SE = 0.19, n = 16), whereas a reduced intensity was seen in invasive carcinoma (mean 2.70; SE = 0.14; n = 67). The difference between these two scores is statistically significant (P = 0.004, t test). EDD/hHYD reactivity decreased with increasing SBR score. Well-differentiated tumors gave a mean stain score of 2.97 (SE = 0.35), indicating little decrease compared with reactivity observed in normal mammary ducts. Moderately differentiated tumors gave a reactivity score of 2.85, and poorly differentiated mammary ductal carcinoma demonstrated a mean staining score of 2.27 (SE = 0.26; P = 0.136, f test). This final score was the lowest observed of the three candidate TSGs, suggesting that EDD/hHYD reactivity was reduced the most of the three TSGs in mammary ductal carcinoma. The reliability estimate for EDD/hHYD was the highest, scoring 0.897, also showing good statistical agreement in the scoring of EDD/hHYD reactivity.

**Mutations and Protein Expression.** We compared the immunohistochemical staining scores for those cases that were evaluated using dHPLC to detect mutations. In all cases, there was a statistically significant association between these two measures; however, the trend of staining score was not the same for each gene (Fig. 6). Those cases with mutations in CSNK1ε had a mean staining score of 2.50 (SE = 0.19) compared with 3.66 (SE = 0.14) in cases with no mutations detected (P < 0.0005). Cases with DLG1 mutations detected had a mean staining score of 2.50 (SE = 0.16) compared with 3.62 (SE = 0.13) observed in those without detectable mutations (P < 0.0005). The mean EDD/hHYD staining score for cases with mutations detected in that gene was 3.69 (SE = 0.30) compared with 2.74 (SE = 0.22) in cases without mutations (P = 0.021, t test).

**LOH and Protein Expression.** We compared the staining score for each protein with the LOH frequency. The mean staining score of CK1ε was reduced in those cases showing LOH for two of the three microsatellite markers flanking the CSNK1ε gene. In all cases, there was a reduced mean staining score for hDlg when LOH was detected in the markers flanking DLG1. EDD/hHYD gave the opposite result. LOH of three microsatellite markers was found to be significantly linked to reduced staining score. They were d22s272 (P = 0.0014, t test), d3s1265 (P = 0.014, t test), and d3s1272 (P = 0.00033, t test).

**Immunohistochemical Reactivity and Clinical Indicators.** To further evaluate the role of DLG1, CSNK1ε, and EDD/hHYD in mammary ductal carcinoma, we looked for association of protein stain of each of these TSGs with known clinical indicators (Table 1). We examined lymph node, ER, PR, p53, Ki67, and ErbB2 status of each...
case studied. We also associated protein stain of the three TSGs and each of the clinical indicators listed above with SBR score. In this evaluation, reduced stain corresponds to cancer cases wherein protein staining intensity was less than that observed in case-specific normal tissue.

Of the tested indicators, CKIe staining showed a significant association only with PR and ER status. The mean CKIe staining score of PR(−) cancers was 2.63 (SE = 0.22) compared with 3.26 (SE = 0.15) in PR(+) cancers (P = 0.02, \( \chi^2 \) test). CKIe reactivity was decreased in 65% (13 of 20) of ER(−) cancers (P = 0.035, t test), and a statistically significant trend of reduced CKIe staining in ER(−) cancers and increased or normal expression in ER(+) cancers was found (cH \( \chi^2 \) = 0.023). hDlg staining showed a statistically significant association only with Ki67 and p53 status. The mean hDlg staining score was 2.28 (SE = 0.18) in Ki67(−) cancers and 3.15 (SE = 0.12) in Ki67(+) cancers (P = 0.00045, t test). The mean hDlg staining score was 2.70 (SE = 0.15) in p53(−) cancers and 3.19 (SE = 0.16) in p53(+) cancers (P = 0.03, t test). EDD/hHYD staining was reduced in both ER(−) and PR(−) cancers (2.44 and 2.53, respectively) compared with ER(+) and PR(+) cancers (2.87 and 2.86, respectively). Of the indicators assayed, SBR score was significantly associated only with ER and PR status. All of the well-differentiated ductal mammary carcinoma cases were ER(+) and 77.7% (14 of 18) of the poorly differentiated cancers were ER(−). This trend was significant (P < 0.0005). Similarly, 91.7% (11 of 12) of the well-differentiated cancers were PR(+) whereas 83.3% (15 of 18) of the poorly differentiated cancers were PR(−). This trend was significant (P < 0.0005).

CKIe and hDlg staining were both reduced in less differentiated cancers. A total of 9.1% (1 of 11) of well-differentiated, 41.7% (15 of 36) of moderately differentiated, and 55% (11 of 20) of poorly differentiated cancers showed reduced CKIe reactivity (P = 0.017, \( \chi^2 \) test for trend). Likewise, reduced hDlg reactivity was found in 25% (3 of 12) of well-differentiated, 40% (14 of 35) of moderately differentiated, and 66.7% (12 of 18) of poorly differentiated ductal mammary carcinoma (P = 0.019). A similar trend for EDD/hHYD was not observed (P = 0.170); however, the mean staining score decreased as the mammary carcinomas became more poorly differentiated.

**DISCUSSION**

The high frequency of somatic mutations we have discovered in CSNK1e, together with mounting evidence that CKIe activates the Wnt pathway, suggests that Wnt pathway activity changes may contribute to at least some cases of breast cancer. The role of Wnt signaling in breast cancer has been uncertain, although Wnt-1 was
originally identified as a mammary oncogene in mice infected with mouse mammary tumor virus (45). The Wnt pathway components APC, β-catenin, and Axin do not show high mutation rates in breast cancer in contrast to the situation with colorectal cancer (46). However, several components of the Wnt signaling pathway, including Wnt-1, β-catenin, and the target genes c-myc and cyclin D1, have been reported as showing elevated expression in breast cancer (47, 48), and high β-catenin activity (indicated by high levels of the protein in the cytoplasm and/or nucleus) is associated with poor prognosis in breast cancer (47). It has therefore been suggested that Wnt pathway activation in breast cancer may result from somatic mutations in other regulators of the pathway that have yet to be discovered or analyzed (46). Our results suggest that CKIε may be such a regulator of the pathway.

In vertebrates, CKIε has been shown to be a positive regulator of Wnt signaling and to be required to transduce Wnt signals. Furthermore, inhibition of endogenous CKIε with a kinase-defective version of the enzyme or with CKIε antisense oligonucleotides attenuates Wnt signaling (49). In mammalian cells, CKIε activates expression of a Lef-1 reporter gene ~10-fold, and this can be inhibited by coexpression of axin (49). These results led to the suggestion that CKIε activates the Wnt pathway by interacting with the β-catenin-Lef-1/TCF complex. It is now known that CKIε regulates the Wnt pathway both positively and negatively, by interacting directly with most of the known components of the pathway (50, 51). The reported apparently opposite effects of CKIε on the Wnt pathway have led to the suggestion (52) that CKIε could have opposite effects in the presence or absence of Wnt signaling. When the pathway is activated by Wnt

Fig. 6. Association between staining reactivity and mutations. Staining score in cases with (right) and without (left) mutations detectable by denaturing high-performance liquid chromatography using the conditions described. The y axis is staining score, and the x axis is case number. A, CKIε; B, Mδg; and C, EDD/hHYD. Mean staining score is indicated by sidebars. There was a statistically significant difference in staining score between cases with and without mutations. Note that staining decreases in cases with mutations in DLG1 and CSNK1ε but increases in cases with mutations in EDD/hHYD.
lignand, CK1ε could contribute positively to activation by phosphorylating and activating Dsh, leading to glycogen synthase kinase-3β (GSK-3β) inactivation, but CK1ε would be unable to exert its inhibitory effect on APC and β-catenin because GSK-3β is also required for this effect. In the absence of Wnt lignand, GSK-3β would remain active and CK1ε could contribute to inactivation of APC and β-catenin by creating priming sites for GSK-3β (52). This dependence of CK1ε action on the presence and activity of GSK-3β has been termed a molecular switch for the Wnt pathway (15).

Mutations in hDlg could also contribute to breast and other cancers by affecting the interactions of this protein with the Wnt signaling pathway. hDlg binds directly to APC, which is an important Wnt pathway component and known colorectal tumor suppressor protein (53). This binding occurs both in vitro and in vivo, and the hDlg/APC complex can block cell cycle progression (25). Another potential interaction of hDlg with the Wnt pathway is suggested by a recent report (54) that hDlg and other members of the PSD-95 subfamily of MAGUKs can bind directly to the Frizzled-1, -2, -4, and -7 receptors, especially in those cases where the marker was physically close to the gene. For CSNK1ε, four of six tumors showing mutations in the gene also showed LOH for both centromeric and telomeric microsatellite markers. For DLG1, two of six tumors showing mutations in the gene also showed LOH for the centromeric marker and two showed LOH for the telomeric marker (see supplementary data published online for details of individual cases). LOH could be selected in tumors either because it removes a dominant tumor-suppressing allele or because it increases the dose of an oncogenic allele. We cannot distinguish between these possibilities in our studies, but the statistically significant association between mutations and LOH does support our conclusion that the mutations are selected during growth of the tumor and are functionally significant.

The presence of somatic mutations in CSNK1ε and DLG1 was associated in a statistically significant manner with decreased antibody reactivity for each gene product. Complete correlations are not expected in these studies because all of the mutations we found are point mutations, which may affect protein activity rather than amount of protein or its reaction with the antibody. The two tumors in which stop codons were detected showed, as expected, very low levels of corresponding protein reactivity, although the antibody against CK1ε recognized a COOH-terminal peptide, whereas the antibody against hDlg recognized an NH₂-terminal peptide.

Our results with EDD/hHYD are different from those with CSNK1ε and DLG1 in several respects. First, 40% (4 of 10) of the mutations detected in EDD/hHYD were synonymous or silent mutations, and the ratio between nonsynonymous and synonymous was not significantly different from that predicted for unselected mutations. Second, all of the nonsynonymous mutations found in EDD/hHYD were missense changes predicting conservative changes in the protein. Third, mutations detected in EDD/hHYD were associated with an increase rather than a decrease in reactivity for the gene product. We interpret these differences as showing that the mutations did not lead to functionally significant protein alterations. However, of the three gene products, EDD/hHYD showed the greatest mean decrease in reactivity in inva-
sive breast cancer, suggesting that changes in its expression may be functionally significant even if genetic changes do not contribute to tumor formation and underscores the possibility that mutations in other regions of EDD/hHYD may be affecting EDD/hHYD expression.

We found that reactivity of each gene product decreased as tumor differentiation decreased and SBR score increased. As the ductal carcinoma became more poorly differentiated, the staining for each protein decreased or was lost completely suggesting that these proteins may be involved in tumor suppression as suggested from the mutant phenotypes in model organisms. We also found that protein reactivity for each gene product increased in DCIS. This phenomenon was most pronounced for CKIε and hDlg.

Associations were found between hDlg stain reactivity and p53 status as well as Ki67 status. We also found that 62.5 and 75% of PR (−) cases showed a decrease in CKIε and EDD/hHYD staining. Similarly, we found that 65 and 75% of ER (−) cases showed a decrease in CKIε and EDD/hHYD staining. As EDD/hHYD was first characterized as a progesterin-regulated gene, it follows that EDD/hHYD reactivity would associate with PR status, but it is not clear why CKIε should show a similar trend. We found no association between hDlg staining and ER/PR status, and no significant association between reactivity of any of the gene products and ErbB2 (Her2/Neu) or lymph node status.

The somatic mutations we discovered in CSNK1ε, DLG1, and EDD/hHYD show that this candidate gene approach can be successful in identifying genes that may be functionally significant in human cancer. At least 50 additional TSGs have been identified in Drosophila through the finding of excess growth caused by loss-of-function mutations. At least 50 additional TSGs have been identified in Drosophila by cDNA expression array. World J. Gastroenterol., 8: 580–585, 2002.


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