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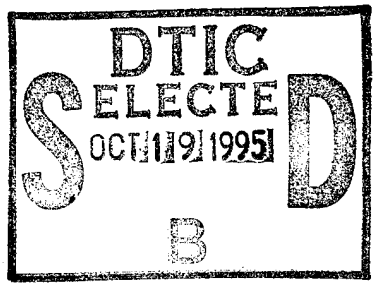

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The studies in this application are directed toward understanding further the nature and clinical significance of somatic mutations in breast cancer. In several tumor types, loss of heterozygosity (LOH, allelic loss) has been shown to inactivate a tumor suppressor gene in the affected chromosomal region. Chromosomes 16q and 18q have been shown to be affected by LOH in 20-50% of breast cancers. Candidate tumor suppressor genes from 16q and 18q are the E-cadherin and DCC (for deleted in colorectal cancer) genes, respectively. Each encodes a transmembrane protein that may function in cell-cell adhesion, and appropriate E-cadherin function is known to be dependent on its interactions with α- and β-catenin. In studies to date, we have shown that alterations in E-cadherin and α- and β-catenin expression are frequent in human breast cancer-derived cell lines, and that in some cases the decreased expression may result from mutations in the genes. Furthermore, the frequent alterations in the expression of these proteins argue that loss of function in the E-cadherin-catenin pathway may be critical in breast cancer pathogenesis. Additional studies should provide new insights into breast cancer pathogenesis. Ultimately, the findings may lead to improved diagnosis and clinical management of patients with breast cancer.

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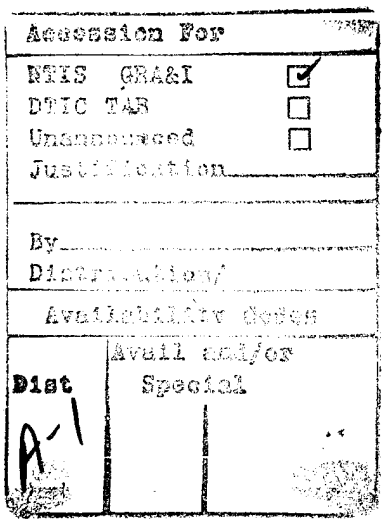

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4. TABLE OF CONTENTS

5. INTRODUCTION

Nature of the Problem

As is the case for many common human cancers, the development of breast cancer is undoubtedly a highly complex process with many potential causes, including hormonal, dietary, and environmental factors and both inherited and somatic mutations. The rationale for the studies we have undertaken and the others proposed in the initial application and this annual report is that further identification and characterization of the genetic alterations in breast cancer development will be useful not only for furthering our understanding of breast cancer pathogenesis, but may also lead to improvements in the diagnosis and management of patients with breast cancer.

Background of Previous Work

A current view is that human cancers arise through a multi-stage evolutionary process driven by inherited and somatic mutation of cellular genes and clonal selection of variant progeny with increasingly more aggressive growth properties (1-3). Three classes of genes - proto-oncogenes, tumor suppressor genes, and DNA repair genes - are targeted by the mutations. The vast majority of the mutations that contribute to the development and behavior of cancer cells are somatic and are present only in the neoplastic cells of the patient. Although only a relatively small subset of all mutations in cancer cells are present in the germline of affected individuals, such mutations not only predispose to cancer, they can be passed on to future generations (4).

Over fifty different proto-oncogenes have been identified through a variety of experimental strategies (1). In general, these genes have critical roles in a variety of growth regulatory pathways, and their protein products are distributed throughout virtually all subcellular compartments. Only a subset of the various proto-oncogenes are targeted by mutations in human cancers. The oncogenic variant alleles present in cancers have gain-of-function mutations resulting from point mutation, chromosomal rearrangement, or gene amplification of the proto-oncogene sequences. While oncogenic alleles harbor activating mutations, tumor suppressor and DNA repair genes are inactivated in human cancer (2-5). A large number of tumor suppressor genes are hypothesized to exist; however, only about a dozen or so have been identified thus far (4). Like proto-oncogenes, the cellular functions of the tumor suppressor genes identified thus far appear to be diverse. The existence of many other tumor suppressor genes is based on indirect evidence, including the frequent loss of specific chromosomal regions in various human tumor types. Usually, the loss involves only one of the two parental chromosomal sets, or alleles, present in normal cells. Thus, the loss is termed an allelic loss or loss of heterozygosity (LOH). In accord with Knudson's hypothesis, allelic losses have been postulated to result in the inactivation of a tumor suppressor gene in the affected chromosomal region (4).

Over the past decade much progress has been made in elucidating some of the oncogene and tumor suppressor gene alterations present in invasive breast cancers. Several oncogenes have been found to be altered in primary cancers and cell lines (6); the *NEU* (also know as *erbB2* or *HER-2*) and cyclin D1 genes appear to be altered most frequently. Somatic mutations in well-characterized tumor suppressor genes, such as the *RB1* and *p53* genes, are present in about 15-20% and slightly less than 50% of cancers, respectively (6). Recent studies also suggest that the *p16* gene on chromosome 9p may be inactivated in upwards of 15-20% of primary breast cancers and cell lines (7). Finally, although the *BRCA*1 gene on chromosome 17q is mutated in the germline of a sizeable fraction of those with inherited breast and/or ovarian cancer, in studies to date, *BRCA1* appears to be infrequently (if ever) affected by somatic mutations in sporadic breast cancers (8,9).

Evidence obtained from LOH studies in breast cancers suggests that only a fraction of the possible specific tumor suppressor gene alterations that may exist have been identified thus far. LOH has been observed on chromosomes 1q, 3p, 11p, 13q, 16q, 17p, 17q, 18q, and 22q in

breast cancers (10-20). Three independent studies have reported that LOH involving chromosome 18q can be observed in 35-70% of breast cancers; the common region on 18q affected by LOH in the majority of cases was localized to 18q21-23 (17,18,20). This region contains the *DCC* (for *deleted in colorectal cancer*) gene, which encodes a large transmembrane protein with similarity to immunoglobulin superfamily cell adhesion molecules (CAMs), including NCAM and L1 (21-23). In colorectal tumors, the *DCC* gene has properties expected of a tumor suppressor gene, including absent or reduced gene expression in the majority of colorectal tumor cell lines and primary tumors (as compared to normal colonic mucosa), LOH at the *DCC* locus in over 70% of cancers, and grossly-detectable somatic rearrangements of the gene in about 15% of cancers (23). In addition, Thompson et al. have reported on studies of *DCC* expression by Northern blot analysis in primary breast cancers. These authors found that 14/34 of breast cancers expressed the normal 12kb *DCC* transcript (20). However, in five of the 14 tumors, an altered 11 kb transcript was also detected. The remaining 20 cases had no detectable *DCC* gene expression. Finally, recent gene transfer studies have demonstrated that exogenous expression of a wild-type *DCC* cDNA will suppress the tumorigenic properties of a human squamous cell line lacking endogenous *DCC* expression. Based on these observations and the prevalence of 18q LOH in breast cancers, the *DCC* gene may be inactivated in a subset of breast cancers and may function as a tumor suppressor gene in breast cancer.

Chromosome 16q LOH has been seen in 30-50% of primary breast cancers (16,19). An interesing candidate tumor suppressor gene from 16q is the *E-cadherin* gene, which endcodes a calcium-dependent homotypic cell adhesion molecule (24). Decreased or undectable levels of E-cadherin expression have been noted in many immunohistochemical studies of epithelial cancers (25-42). In some tumor types, decreased or absent E-cadherin expression has been associated with loss of differentiated features. In addition, the loss of E-cadherin expression in some cancers has been found to correlate with an increased likelihood of distant metastasis in the patient, suggesting a role for *E-cadherin* as an invasion or metastasis suppressor gene. Indeed, several in vitro studies have demonstrated that loss of E-cadherin function in cell lines is correlated with the acquisition of more invasive properties. Somatic mutations in the *E-cadherin* gene have been identified in some gastric carcinomas (43,44), particularly diffuse type gastric cancers, and somatic missense and nonsense mutations in *E-cadherin* were found in a small subset of the 130 endometrial and ovarian tumors studied (45). Finally, in some breast cancer lines and a prostate cancer cell line, evidence has been obtained that altered transcriptional regulation may account for loss of *E-cadherin* expression (46,47). Nevertheless, in the majority of breast and other cancers where decreased or absent *E-cadherin* expression has been observed in immunohistochemical studies, the mechanisms underlying its altered expression remain poorly understood.

Appropriate E-cadherin function is dependent upon its ability to link to the submembrane cytoskeletal matix through interactions with proteins termed the catenins (48-58). The catenins include α-catenin, a protein with similarity to the actin-binding protein vinculin; β-catenin, a relative of the *Drosophila* armadillo protein which functions in the determination of segment polarity; and γ-catenin, which is identical to plakoglobin and is found in both adherens junctions and desmosomal junctions. Functional interactions between epithelial cells may be abrogated not only by defects in E-cadherin structure or expression, but also be alterations in catenin expression or structure (40,59-61).

Purpose of Present Work and Methods of Approach

In summary, data from LOH studies suggest chromosomes 16q and 18q may harbor tumor suppressor genes which are inactivated in primary breast cancers. Candidate suppressor genes from these chromosomes are the *E-cadherin* and *DCC* genes. Both encode transmembrane molecules that may function in cell-cell inteactions and/or the transduction of growth regulatory signals. As reviewed above, preliminary data suggest that alterations in the expression of both genes are present in breast cancers. The proposed studies will determine the prevalence and nature of chromosome 16q and 18q and *E-cadherin* and *DCC* alterations in breast cancer.

Moreover,understanding the relationship of these alterations to the pathogenesis of breast cancer is a major goal of our studies. Ultimately, it is hoped that the insights obtained from such studies can be applied to improving the diagnosis and management of breast cancer patients.

In the original application, three technical objectives were proposed to accomplish the proposed goals:

1) To determine the frequency of LOH affecting chromosomes 16q and 18q in primary breast cancers; to identify the common region affected by LOH on each of these chromosomes; and to identify the possible association of such LOH events with clinical and histopathological features.

2) To identify specific genetic alteations in the *DCC* and *E-cadherin* genes in breast cancers.

3) To address the functional role of the *DCC* and *E-cadherin* genes as tumor suppressor genes in breast cancer.

6. BODY

As noted in the original application, previous studies of LOH in breast cancers have provided evidence that chromosomes 16q and 18q each may be altered at relatively high frequency in primary tumors. Moreover, preliminary evidence of alterations in *E-cadherin* and *DCC* expression in breast cancers have been obtained and reported by others. Hence, we proposed to carry out studies to identify specific alterations in the *E-cadherin* and *DCC* genes (technical objective two) concurrently with the LOH studies (technical objective one). Although experimental studies to address technical objective one remain a major goal of the application, we decided to enter first into studies to address technical objective two. These experimental studies will be summarized here and additional information is provided in the Pierceall et al. reference (62) included in the appendix.

Frequent alterations in E-cadherin and α- and β-catenin expression in human breast cancer cell lines

We carried out studies of *E-cadherin*, *α-* and *β-catenin* expression in eighteen breast cancer cell lines to determine the prevalence and nature of alterations in these genes in breast cancer. Ten lines failed to express E-cadherin protein at detectable levels in enhanced chemiluminesence (ECL)-Western blot assays. As assessed by a ribonuclease (RNase) protection assay, seven of the eighteen lines failed to produce detectable levels of *E-cadherin* transcripts. In one line (SK-BR-3) lacking both *E-cadherin* transcripts and protein, a homozygous deletion of a large portion of the *E-cadherin* gene was noted. Localized sequence alterations in *E-cadherin* transcripts were not identified in any lines. In contrast to the results of a previous study, no relationship was identified between *E-cadherin* expression and HER-2/NEU expression. Two of the eighteen lines had no detectable α-catenin protein and six others had reduced levels. The two lines lacking α-catenin protein had reduced or undetectable levels of *α-catenin* transcripts, while no consistent changes in *α-catenin* transcript levels were seen in the lines with reduced, but detectable levels of α-catenin protein. Similarly, although two lines lacked β-catenin protein, in most lines the levels of *β-catenin* transcripts and protein were not well correlated with one another. ECL-Western blot studies of the expression of three other proteins associated with the membrane cytoskeletal matrix - β-fodrin (βII-spectrin), the α subunit of Na^+-K^+-ATPase, and band 4.1 - failed to provide any evidence that the altered E-cadherin and catenin expression was simply representative of a generalized change in the expression of proteins associated with the membrane cytoskeletal matrix in the affected cell lines. Hence, our findings suggest that alterations in *E-cadherin* and *α-* and *β-catenin* expression are a frequent and a relatively specific change in human breast cancer-derived cell lines. In addition, in some cases the decresased expression may result from mutations in the genes. Furthermore, the frequent alterations in the expression of these proteins argue that loss of function in the E-cadherin-catenin pathway may be critical in the development of many breast cancers. These studies are described in detail in the manuscript by Pierceall et al. that is currently in press at Oncogene (ref. 62 and appendix).

Mechanisms underlying altered *E-cadherin* expression in breast cancers

As reviewed above, many of the breast cancer cell lines studied had reduced or undetectable levels of *E-cadherin* gene and protein expression. While in some cases, such as the SK-BR-3 cell line, mutations in the *E-cadherin* gene account for loss of *E-cadherin* expression, the mechanisms underlying the diminished levels of *E-cadherin* gene and protein expression noted in nearly half of the lines remain poorly understood. Although the possible mechanisms include localized mutations in the *E-cadherin* gene that interfere with synthesis, processing, or stability of its transcripts, *E-cadherin* expression may, in fact, be decreased as a result of specific defects in upstream regulatory pathways or transcription factors that control its expression. Indeed, consistent with this notion, previous studies from another group suggest that *E-cadherin* promoter activity may be correlated with endogenous *E-cadherin* expression in some breast cancer cell lines (46).

It should be noted that the prior studies of *E-cadherin* promoter activity in breast cancer cell lines utilized plasmid constructs generated with mouse *E-cadherin* promoter sequences (46). Therefore, in an effort to characterize the sequences responsible for (human) *E-cadherin* promoter activity in breast cancer cells we have obtained phage clones containing human E-cadherin exon 1 and 2, as well as more than 5 kilobases (kb) of sequences upstream of the presumptive *E-cadherin* transcriptional start site. Over 1.5 kb of genomic DNA sequences located 5' to the *E-cadherin* translational start site (ATG) has been sequenced in their entirety. Eight different luciferase report contructs have been generated containing various *E-cadherin* 5' genomic sequence fragments. These constructs will be tested for their promoter activity in breast cancer cell lines with and without endogenous E-cadherin expression. Specifically, we will identify the sequences responsible for directing luciferase reporter gene expression in cell lines with endogenous *E-cadherin* expression. Similarly, we will charaterize the promoter sequences that might be responsible for repressing luciferase gene expression in lines lacking endogenous *E-cadherin* expression. Subsequently, gel shift and "foot-printing" studies will be carried out to distinguish between the two most likely explanations for the promoter activity results: i) loss of *E-cadherin* expression as a result of the absence of a transcriptional activation activity; or ii) loss of *E-cadherin* expression as a result of the presence of a novel repressor activity. Ultimately, we hope to characterize some of the transcription factors, and perhaps even some of the signalling pathways, responsible for controlling *E-cadherin* expression in normal and neoplastic breast cells.

Mechanisms underlying altered *α*- and *β-catenin* expression in breast cancers

Appropriate E-cadherin function is dependent upon its interaction with other proteins, including α- and β-catenin (48-61). As noted above, a number of the breast cancer cell lines we studied had reduced or undetectable levels of α- and/or β-catenin expression (62). While the levels of E-cadherin protein and transcripts were relatively concordant in the cell lines, the relative levels of catenin gene and protein expression were often very discordant. Missense, nonsense, or frameshift mutations in catenin transcripts might account for these observations. In an effort to address these possibilities, we plan to carry out polymerase chain reaction (PCR)-based studies of the sequence of *α*- and *β-catenin* transcripts in breast cancer cell lines with reduced or undetectable levels of the corresponding catenin protein. We will also carry out studies of *α*- and *β-catenin* transcript and protein half-lives in a number of the cell lines to determine if post-transcriptional and/or post-translational mechanisms underlie the discordancies observed between the relative abundance of catenin transcripts and protein. In addition, immunohistochemical studies to determine the prevalence of altered α- and β-catenin expression (as well as E-cadherin expression) in primary breast cancers are underway. The relationship between altered E-cadherin and catenin expression and clinical and histopathological features will be addressed in the cancers.

Alterations in *DCC* expression and gene structure in breast cancers

These studies are underway, and thus far we have found that *DCC* gene and protein expression is below the levels of detection of RNase protection and ECL-Western blotting, respectively, in primary breast cancers and cell lines. At present, further definitive studies of DCC expresion are stymied in large part by the currently available anti-DCC antibody reagents. Additional studies of DCC protein expression in primary breast cancers are critically dependent upon the generation of improved antibody reagents for the detection of DCC protein in immunohistochemical studies. The generation of new and improved polyclonal and monoclonal anti-DCC antibodies for such studies should markedly improve our ability to characterize DCC expression in primary breast cancers. Following our studies of DCC expression we will determine the mechanisms underlying loss of DCC expression in breast cancers, using the approaches and techniques reviewed in our initial application.

Chromosome 16q and 18q LOH analyses

These studies have not yet been undertaken, because of our increased emphasis on studies of *E-cadherin*, *α*- and *β-catenin*, and *DCC* expression and gene structure in breast cancers. Should we fail to obtain compelling evidence for *E-cadherin* and *DCC* inactivation in breast cancers, we

plan to embark on comprehensive studies to define the prevalence and chromosome regions affected by 16q and 18q LOH in breast cancers.

Identification of tumor suppressor gene function for the *E-cadherin* and *DCC* genes in breast cancer

As noted in the original application, these studies were not scheduled to begin until year 2. The first phase of these studies - the characterization of *DCC* and *E-cadherin* expression in the breast cancer cell lines - has been completed. In addition, we have generated the requisite *DCC* and *E-cadherin* gene constructs for the studies. In years 2-3, we plan to undertake transfection, isolation, and prelimary characterization of breast cancer cell lines expressing *DCC* and *E-cadherin* cDNAs. In addition, in cell lines lacking catenin expression, we will carry out transfection with the appropriate catenin cDNA(s).

7. CONCLUSIONS

Frequent alterations in *E-cadherin* and α- and β-*catenin* expression in human breast cancer cell lines

In summary, the data reviewed above (and presented in detail in the Pierceall et al. manuscript - see appendix) demonstrate that alterations in *E-cadherin* and α- and β-*catenin* are common in breast cancer cell lines. Although we cannot exclude the possibility that alterations in E-cadherin and catenin expression and gene structure may have arisen during the establishment and passage of the cell lines, the prevalence of altered expression of E-cadherin and catenin in the breast cancer cell lines appears to be relatively well correlated with the results of immunohistochemical studies of primary breast cancers. As described above, we plan to undertake additional experimental studies to characterize the apparently complex assortment of mutational and altered regulatory mechanisms underlying the alterations in E-cadherin and catenin expression in breast cancer cells. Finally, our studies have provided further support for the proposal that defects in the E-cadherin-catenin pathway may be a critical and necessary step in the generation of advanced breast cancer cells. Additional experimental support for this proposal will be obtained by our gene transfer studies in which we directly examine the role of *E-cadherin* and α- and β-*catenin* cDNAs in suppressing the altered phenotypic properties of breast cancer cells.

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PERSONNEL WITH SALARY SUPPORT

Name	Role on Project	% Effort on Project
Fearon, Eric R.	Principal Investigator	10
Rimm, David L.	Co-investigator	10
D'Aquila, Thomas	Research Assistant	100

9. APPENDIX

1 Manuscript (8 pages):
Pierceall, W. E., Woodard, A. S., Morrow, J. S., Rimm, D., and Fearon, E. R.: Frequent alterations in E-cadherin and α- and β-catenin expression in human breast cancer cell lines. Oncogene, in press, 1995.

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Frequent alterations in E-cadherin and α- and β-catenin expression in human breast cancer cell lines

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Alterations in intercellular junction and membrane cytoskeletal proteins may underlie some of the morphological, invasive and metastatic properties of cancer. E-cadherin, a transmembrane protein that functions in epithelial cell-cell interactions at adherens junctions, is linked to the membrane cytoskeletal matrix through interactions with α- and β-catenin. We have carried out studies of *E-cadherin* and α- and β-*catenin* in 18 breast cancer cell lines to determine the prevalence and nature of alterations in these genes in breast cancer. Ten lines failed to express E-cadherin protein at detectable levels and seven failed to produce detectable levels of *E-cadherin* transcripts. In a line lacking E-cadherin expression (SK-BR-3) a homozygous deletion of a large portion of the *E-cadherin* gene was noted. Localized sequence alterations in *E-cadherin* transcripts were not identified in any lines. In contrast to the results of a previous study, no relationship was identified between *E-cadherin* expression and HER-2/NEU expression. Two of the 18 lines had no detectable α-catenin protein and six others had reduced levels. The two lines lacking α-catenin protein had reduced or undetectable levels of α-*catenin* transcripts, while no consistent changes in α-*catenin* transcript levels were seen in the lines with reduced, but detectable, levels of α-catenin protein. Similarly, although two lines lacked β-catenin protein, in most lines the levels of β-*catenin* transcripts and protein were not well correlated with one another. Our findings suggest that alterations in E-cadherin and α- and β-catenin expression are frequent in human breast cancer-derived cell lines, and that in some cases the decreased expression may result from mutations in the genes. Furthermore, the frequent alterations in the expression of these proteins argue that loss of function in the E-cadherin-catenin pathway may be critical in the development of many breast cancers.

Keywords: breast cancer; E-cadherin; α-catenin; β-catenin; cytoskeleton; cell-cell interactions

Introduction

The cadherins are a family of Ca^{2+}-dependent transmembrane proteins which appear to mediate cell-cell interactions through homotypic interactions (Takeichi, 1991; Tsukita *et al.*, 1992). E-cadherin is expressed on epithelial cells and its function depends upon the ability of its cytoplasmic sequences to link to the submembrane cytoskeletal matrix through interactions with proteins termed the catenins (Nagafuchi and Takeichi, 1988; Ozawa *et al.*, 1989; 1990; Kintner, 1992). The catenins include α-catenin, a protein with similarity to the actin-binding protein vinculin (Nagafuchi *et al.*, 1991; Herrenknecht *et al.*, 1991); β-catenin, a relative of the *Drosophila* armadillo protein which functions in the determination of segment polarity (McCrea *et al.*, 1991; Pfiefer and Wiechaus, 1990; Butz *et al.*, 1992) and γ-catenin, which is identical to plakoglobin and is found in both adherens junctions and desmosomal junctions (Francke *et al.*, 1989; Knudsen and Wheelock, 1992; Hinck *et al.*, 1994; Nathke *et al.*, 1994). Functional interactions between epithelial cells may be abrogated not only by defects in E-cadherin structure or expression, but also by alterations in catenin expression or structure (Hirano *et al.*, 1992).

Decreased or undetectable levels of E-cadherin expression have been noted in many immunohistochemical studies of epithelial cancers (Schipper *et al.*, 1991; Shimoyama and Hirohashi, 1991a,b; Shiozaki *et al.*, 1991; Umbas *et al.*, 1992; Brabant *et al.*, 1993; Bringuier *et al.*, 1993; Doki *et al.*, 1993; Dorudi *et al.*, 1993; Gamallo *et al.*, 1993; Mayer *et al.*, 1993; Moll *et al.*, 1993; Morton *et al.*, 1993; Oka *et al.*, 1993; Rasbridge *et al.*, 1993; Kadowaki *et al.*, 1994; Rimm *et al.*, 1995). In some tumor types, the loss of E-cadherin expression has been associated with loss of differentiated features in the tumor. In addition, the loss of E-cadherin expression in some cancers has been found to correlate with an increased likelihood of distant metastasis in the patient, suggesting a potential role for E-*cadherin* as an invasion or metastasis suppressor gene. More direct experimental support for this proposal has been obtained from in vitro studies of several rodent and human tumor cell lines in which loss of E-cadherin function is correlated with the acquisition of invasive properties (Behrens *et al.*, 1989; Frixen *et al.*, 1991; Vleminckx *et al.*, 1991; Birchmeier *et al.*, 1993).

Further evidence that loss of E-cadherin function may be critical to tumorigenesis has been provided by other studies. The chromosome 16q region containing the E-*cadherin* gene is affected by loss of heterozygosity (LOH) in breast and prostate cancers (Sato *et al.*, 1990; Bergerheim *et al.*, 1991; Carter *et al.*, 1991; Lindblom *et al.*, 1993). Somatic mutations in the E-*cadherin* gene have been identified in some gastric carcinomas, particularly diffuse type gastric cancers (Becker *et al.*, 1994; Oda *et al.*, 1994). In addition, a small subset of 130 endometrial and ovarian tumors studied were found to have somatic missense and nonsense mutations in the E-*cadherin* coding region (Risinger *et al.*, 1994). Finally, in some breast cancer lines and a

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prostate cancer cell line, evidence has been obtained that altered transcriptional regulation may account for loss of E-*cadherin* expression (Behrens *et al.*, 1991; Bussemakers *et al.*, 1994). Nevertheless, in the majority of cancers where altered E-*cadherin* expression has been observed in immunohistochemical studies, the mechanisms underlying its altered expression remain poorly understood.

Alterations in the catenins have also been seen in some human cancers, Decreased or absent α-catenin expression has been noted in some primary breast, esophageal, and prostate cancers (Shimoyama *et al.*, 1992; Morton *et al.*, 1993; Kadowski *et al.*, 1994). Genetic alterations at the α-*catenin* locus may account for decreased expression in a subset of cases. One of seven prostate cancer cell lines examined had a homozygous deletion of α-*catenin* sequences (Morton *et al.*, 1993) and a lung cancer cell line has been found to have a complete loss of α-catenin expression as a result of localized mutations in both α-*catenin* alleles (Oda *et al.*, 1993). In addition, alterations in β-catenin and plakoglobin expression and phosphorylation have also been noted in some tumor cell lines (Sommers *et al.*, 1994). Furthermore, the protein product of the adenomatous polyposis coli (*APC*) tumor suppressor gene is known to complex with α- and β-catenin, but not with E-cadherins (Rubinfeld *et al.*, 1993; Su *et al.*, 1993; Hulsken *et al.*, 1994). Although the functional significance of the interactions between α- and β-catenin and the APC protein is not yet well understood, the critical role of the APC gene product in tumor suppression in epithelial cells in the gastrointestinal tract is well established (Groden *et al.*, 1991; Nishisho *et al.*, 1991). The interaction between the catenins and an established tumor suppressor gene product lends further support to the proposal that alterations in E-cadherin and catenin function may have a critical role in tumorigenesis.

In the studies described here we have sought to characterize the prevalence of alterations in E-cadherin and α- and β-catenin expression in human breast cancer-derived cell lines and to address the mechanisms underlying their altered expression. We have chosen to examine cell lines in our initial analysis to eliminate ambiguities in studies of protein and RNA expression that might have arisen as a result of the normal cells that are often admixed with neoplastic cells in primary breast cancer specimens. Complete loss or markedly decreased expression of E-cadherin and α-and β-catenin is frequently seen in breast cancer cell lines. The altered expression of E-cadherin and the catenins may result from specific mutations in the genes in some tumors and in others may reflect changes in transcriptional or post-transcriptional regulatory mechanisms. Collectively, the data support the proposal that loss of function in the cadherin-catenin pathway may play a critical role in the pathogenesis of human breast cancer.

Results

Western blot studies of E-cadherin and α- and β-catenin expression

Western blot analyses of E-cadherin, α-catenin and β-catenin expression were carried out on 18 breast carcinoma -derived cell lines (summarized in Table 1). E-cadherin protein was detectable in eight lines, and α-catenin protein was detected at varying levels in 16 lines (Figure 1). No detectable α-catenin protein was seen in two cell lines (lanes 8 and 17). Reduced levels of α-catenin were seen in six lines (lanes 3, 6 and 11–14). β-catenin protein was detected at varying levels in 16 of the cell lines. No detectable β-catenin was seen in two lines (lanes 3 and 14), and decreased levels were noted in six other cell lines (lanes 2, 8, 9, 11–13, 18). In addition, a reactive protein with aberrant migration that may represent a proteolytic breakdown product or a truncated, mutant β-catenin protein product was detected in one line (lane 10).

In an effort to determine if other proteins associated with the membrane cytoskeletal matrix might also display altered expression in the breast carcinoma-derived cell lines, we studied the expression of three

Table 1 E-cadherin, α-catenin, and β-catenin gene and protein expression in breast cell lines

		E-cadherin		*α-catenin*		*β-catenin*		*HER2/Neu*
Cell line#	*Identity*	*RNA*	*Pro*	*RNA*	*Pro*	*RNA*	*Pro*	*Pro*
1	MDA-MB-361	+ + +	+	+ +	+ + +	+ + +	+ + +	+ + +
2	BT-474	+ +	+ +	+ +	+ + +	+	+	+ + + +
3	ZR-75-30	+/–	–	+ + +	+	+ +	–	+ + + +
4	BT-20	+ +	+	+ +	+ + +	+ + +	+ + +	+
5	HBL-100	–	–	+ +	+ + +	+ +	+ +	+
6	DU4475	+	–	+	+	+ + +	+ + +	+/–
7	HS 578T	–	–	+ +	+ + +	+ +	+ + +	+
8	MDA-MB-468	+	+	+	–	+ + +	+	+
9	ZR-75-1	+ +	+ +	+ +	+ + +	+ +	+	+ +
10	BT-483	+ +	+ +	+ +	+ + +	+ +	+ +	+ +
11	MDA-MB-435S	–	–	+	+	+ +	+	+
12	MDA-MB-231	–	–	+ +	+	+ +	+	+
13	MDA-MB-453	+/–	–	+ +	+	+ +	+	+ + +
14	SK-BR-3	–	–	+ + +	+	+ +	–	+ + +
15	T-47D	+ +	+ +	+ +	+ + +	+ +	+ + +	+
16	BT-549	–	–	+ +	+ + +	+ +	+ + +	+
17	MDA-MB-157	–	–	–	–	+ +	+ +	+
18	MCF-7	+ + +	+	+ +	+ + +	+ +	+	+

Relative levels of RNA and protein expression are indicated based on RNase protection studies of gene expression and ECL-Western blot studies of protein expression, with the following scoring system: '–' no detectable expression; '+/–' very low expression; '+' low expression; '+ +' moderate expression; '+ + +' high expression; '+ + + +' very high expression

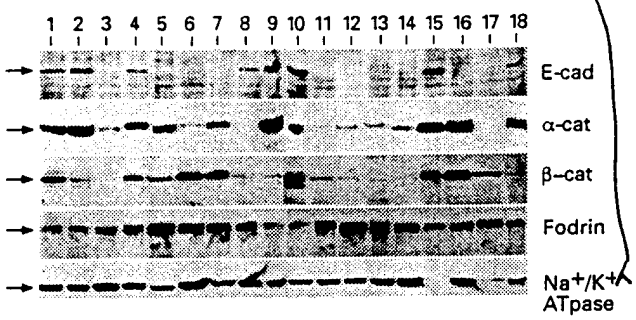

Figure 1 ECL-Western blot studies of E-cadherin and α- and β-catenin expression. Protein lysates were prepared from 18 breast cancer cell lines and 40 μg of protein from each line was loaded for SDS–PAGE on 7.5% gels. Proteins were transferred to Immobilon P membranes, and the membranes were incubated with a specific primary antiserum against E-cadherin, α-catenin, β-catenin, β-fodrin, or Na^+-K^+-ATPase and horesradish peroxidase-labeled secondary antibody reagents. Antibody complexes were detected by ECL. For each protein species studied (e.g., E-cadherin, α-catenin, etc.), although the lysates were electrophoresed on two gels and transferred to two membranes, the ECL exposure times for all 18 lanes are equivalent. The lane numbers correspond to the reference numbers in Table 1

other proteins localized to either the membrane (i.e., α-subunit of Na^+-K^+-ATPase) or the submembrane cortical cytoskeleton (i.e., βII-spectrin or β-fodrin, a spectrin-related protein that complexes with ankyrin and the band 4.1 protein which is thought to stabilize interactions between spectrin and actin). Only relatively subtle differences in β-fodrin levels were noted among the cell lines and most differences appeared to be due to decreased transfer of the gel lanes near the edges of the membrane for this large protein with a relative molecular mass greater than 200 000 (Figure 1 and data not shown). In addition, no apparent differences were noted in the expression of the band 4.1 protein in the cell lines (data not shown). However, two lines were found to have markedly reduced Na^+-K^+-ATPase α-subunit expression (lanes 15 and 17).

RNase protection studies of E-cadherin *and* α- *and* β-catenin *gene expression*

Ribonuclease (RNase) protection studies were carried out in an effort to determine if the levels of E-*cadherin* and α- and β-*catenin* transcripts were correlated with their levels of protein expression in the cell lines. Antisense riboprobes for each gene as well as a control gene (γ-*actin*) were synthesized and incubated with total RNA from each of the 18 cell lines. E-*cadherin* transcripts were detected in 11 lines (Figure 2A). Overall, the relative levels of E-*cadherin* transcripts and protein in the cell lines were well correlated (Table 1, $P < 0.01$). Altered transcriptional regulation and/or mutations interfering with gene expression are likely to account for the concordant decrease in levels of E-*cadherin* transcripts and protein in the affected cell lines.

In contrast to the findings for E-cadherin, the abundance of α- and β-*catenin* transcripts in the cell lines was not well-correlated with their respective protein levels. For example, several cell lines (e.g., lanes 3, 12 and 14) had levels of α-*catenin* transcripts comparable to other lines (Figure 2B), but had very reduced levels of α-catenin protein in the Western blot analysis (Figure 1 and Table 1). The two cell lines lacking α-catenin protein by Western blot analysis had reduced or undetectable levels of α-*catenin* transcripts in the RNase protection analysis (Figure 2B, lanes 8 and 17, respectively). β-catenin protein levels were also not well correlated with transcript levels (e.g., lanes 3, 8, 12, 14 in Figures 1 and 2C; Table 1).

Southern blot and PCR-SSCP analysis of E-cadherin *gene sequences*

To determine if gross rearrangements of E-*cadherin* sequences might account for decreased or undetectable levels of E-*cadherin*, we carried out Southern blot analysis on EcoRI-digested DNA from the cell lines using a full-length E-*cadherin* cDNA probe. Changes in the migration or relative intensity of the detected fragments were seen in two cell lines (SK-BR-3, Figure 3, lane 14; and MDA-MB-468, data not shown). Based on the EcoRI fragments that failed to react with the full-length E-*cadherin* cDNA probe in the analysis of SK-BR-3, the majority of the E-*cadherin* exons in this cell line are affected by homozygous deletion (Figure 3 and data not shown). Consistent with these results, no E-cadherin protein or transcripts were detected in the SK-BR-3 line (Figures 1 and 2A, lane 14). Using an E-*cadherin* cDNA probe corresponding to exon 13–16 sequences in Southern blot analysis, we noted an EcoRI fragment with altered migration in the MDA-MB-468 cell line (data not shown). This cell line

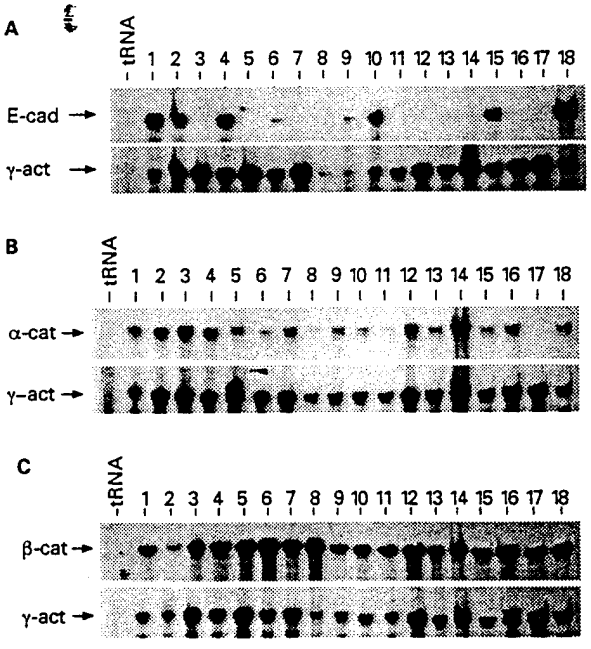

Figure 2 Ribonuclease (RNase) protection assays of E-*cadherin*, α-*catenin* and β-*catenin* gene expression. The lane numbers correspond to the reference numbers for the cell lines in Table 1 and Figure 1, with the exception of a control sample of yeast tRNA. Approximately 5 μg of total RNA from each sample was incubated with 2.5×10^4 c.p.m. of each of the acrylamide/urea gel-purified ^{32}P-labeled anti-sense riboprobes: (**A**) - E-*cadherin* and γ-*actin*; (**B**) - α-*catenin* and γ-*actin*; (**C**) - β-*catenin* and γ-*actin*. The γ-*actin* riboprobe was co-incubated with each of the other riboprobes to control for sample loading and RNA integrity

expressed decreased but roughly concordant levels of E-*cadherin* transcripts and protein. Thus, the altered EcoRI-pattern seen in MDA-MB-468 following Southern analysis with E-*cadherin* cDNA probes is likely to reflect DNA polymorphism rather than mutation. No gross alterations were seen in any of the lines when a full-length α-*catenin* cDNA probe was used for Southern blot analysis of EcoRI-digested DNA from the cell lines (Figure 3 and data not shown).

Because localized mutation in E-*cadherin* sequences have previously been observed in a subset of gastric carcinomas, as well as in some ovarian and endometrial cancers, we carried out a combined polymerase chain reaction and single strand conformational polymorphism (PCR-SSCP) analysis of E-*cadherin* cDNAs obtained from cell lines in which E-*cadherin* transcripts were detected by the RNase protection assay. No sequence alterations were detected in this analysis, suggesting that localized mutations in the E-*cadherin* gene are not common in breast carcinoma cell lines expressing E-*cadherin* transcripts and protein.

Relationship between HER-2/NEU overexpression and E-cadherin expression in breast cancer cell lines

HER-2/NEU overexpression and/or amplification has been noted in a subset of breast cancers, and in several studies overexpression and/or amplification has been shown to be an independent risk factor for disease recurrence (Slamon *et al.*, 1987; Press *et al.*, 1993; Dougall *et al.*, 1994). A recent study has suggested that overexpression of HER-2/NEU in an immortalized human mammary epithelial cell line is associated with an inhibition of E-*cadherin* transcription (D'souza and Taylor-Papadimitriou, 1994). Therefore, we sought to determine if there was a correlation between the levels of HER-2/NEU expression and E-*cadherin* expression in the breast carcinoma-derived cell lines. The relative levels of HER-2/NEU expression were characterized in the cell lines by an ECL-Western blotting approach. We found that there was no apparent correlation between E-*cadherin* expression and HER-2/NEU expression levels (Table 1 and data not shown). It has also been previously reported that E-cadherin expression was reduced but detectable in the SK-BR-3 line, a line with HER-2/NEU amplification and overexpression (D'souza and Taylor-Papadimitriou, 1994). While we confirmed that SK-BR-3 expressed high levels of HER-2/NEU, as we noted above, SK-BR-3 failed to express E-*cadherin* RNA and protein endogenously because of a homozygous deletion involving a large portion of the E-*cadherin* coding sequences.

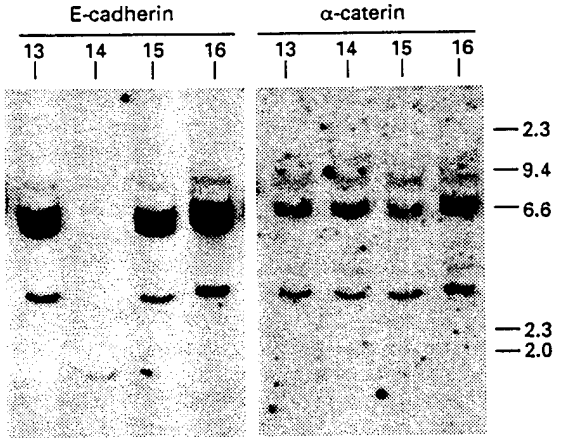

Figure 3 Southern blot analysis of EcoRI-digested breast cancer cell line DNAs with E-*cadherin* and α-*catenin* probes. The lane numbers correspond to the reference numbers for the cell lines in Table 1 and Figure 1. Approximately 10 μg of DNA from each cell line was digested with EcoRI and Southern blot analysis was carried out. The same blot was hybridized to full-length E-*cadherin* and α-*catenin* cDNAs (left and right, respectively). The SK⁻ BR-3 cell line lost most of the EcoRI fragments detected by the E-*cadherin* cDNA probe. The migration of λ/HindIII size markers in kilobasepairs is indicated at the right

Discussion

Proper inter-cellular interactions are critical to the maintenance of normal cell morphology, differentiation, and growth control. Destabilization or loss of normal cell-cell interactions, as a result of defects in the function of the adherens junction or the submembrane cortical cytoskeleton, may have a critical role in the altered phenotype properties of cancer cells. Data supporting this proposal include the following: (i) the observations of decreased or absent E-cadherin or α-catenin reactivity in immunohistochemical studies of a number of different cancers (Schipper *et al.*, 1991; Shimoyama and Hirohashi, 1991a,b; Shiozaki *et al.*, 1991; Umbas *et al.*, 1992; Brabant *et al.*, 1993; Bringuier *et al.*, 1993; Dorudi *et al.*, 1993; Doki *et al.*, 1993; Gamallo *et al.*, 1993; Mayer *et al.*, 1993; Moll *et al.*, 1993; Morton *et al.*, 1993; Oka *et al.*, 1993; Rasbridge *et al.*, 1993; Kadowaki *et al.*, 1994; Rimm *et al.*, 1995); (ii) the identification of mutations in the E-*cadherin* gene in a subset of gastric and gynecologic cancers (Becker *et al.*, 1994; Oda *et al.*, 1994; Risinger *et al.*, 1994), and α-*catenin* mutations in a prostate cancer and a lung cancer cell line (Morton *et al.*, 1993; Oda *et al.*, 1993); (iii) the interaction of a known tumor suppressor gene product, APC, with α- and β-catenin (Rubinfeld *et al.*, 1993; Su *et al.*, 1993; Hulsken *et al.*, 1994); (iv) the demonstration that β-catenin is phosphorylated on tyrosine residues either directly or indirectly by known oncogene products, including src, the EGF receptor and met (Hulsken *et al.*, 1994); and (v) the demonstration that a transfected E-*cadherin* gene can suppress the invasive properties of some tumor cell lines with decreased or absent endogenous E-cadherin expression (Vleminckx *et al.*, 1991).

In the studies described here we have addressed the prevalance of and mechanisms underlying altered E-cadherin and α- and β-catenin expression in breast cancer cell lines. Although previous immunohistochemical studies have demonstrated that E-cadherin and α-catenin immunoreactivity are each decreased or absent in about 50% of primary breast cancers (Shiozaki *et al.*, 1991; Gamallo *et al.*, 1993; Moll *et al.*, 1993; Oka *et al.*, 1993; Rasbridge *et al.*, 1993; Rimm *et al.*, 1995), the mechanisms underlying the altered patterns of immunoreactivity remain quite poorly characterized. In addition, while immunofluorescence studies have been carried out to address β-catenin expression in breast cancer cell lines (Sommers *et al.*, 1994), no studies

addressing β-catenin immunoreactivity or expression in primary breast cancers have been presented in the literature. We have chosen to investigate the mechanisms underlying altered E-cadherin and catenin expression in breast cancer cell lines, because of some of the technical difficulties encountered in detailed studies of protein and RNA expression and gene structure and sequence in many primary breast cancers.

As summarized in Table 1, many lines had reduced or undetectable levels of expression of E-cadherin and/or the catenins. In some lines specific mutations are likely to account for the altered expression patterns. For example, a homozygous deletion of a large portion of the E-*cadherin* gene was noted in one cell line. Nevertheless, with the exception of this line, the mechanisms underlying the diminished levels of E-*cadherin* gene and protein expression noted in nearly half of the lines remain relatively poorly understood. Although the possible mechanisms include localized mutations in the E-*cadherin* gene that interfere with synthesis, processing, or stability of its transcripts, E-*cadherin* expression may be decreased as a result of specific defects in upstream regulatory pathways or transcription factors that control its expression. Indeed, consistent with this notion, previous studies suggest that E-*cadherin* promoter activity may be correlated with endogenous E-*cadherin* expression in some breast cancer cell lines (Behrens *et al.*, 1991). Specific factors regulating E-*cadherin* promoter activity have not yet been well defined. Although a recent study suggested HER-2/NEU overexpression in an immortalized, non-tumorigenic breast cell line was associated with inhibition of E-*cadherin* transcription (D'souza and Taylor-Papadimitriou, 1994), in our studies, we were unable to demonstrate a correlation between HER-2/NEU overexpression and E-cadherin expression in the breast cancer cell lines.

While the relative abundance of E-*cadherin* transcripts and protein correlated well in the cell lines (Table 1), the relative levels of catenin gene and protein expression were often discordant. Specifically, although two lines with no detectable α-catenin protein expressed very reduced or undetectable levels of α-*catenin* transcripts, the majority of lines with reduced α-catenin protein had no consistent differences in α-*catenin* transcript levels. Similarly, most lines with reduced but detectable levels of β-catenin protein had no clear-cut differences in the abundance of β-*catenin* transcripts when compared to lines with abundant levels of β-catenin protein. Given that previous studies have identified α-*catenin* mutations in some cancer cell lines (Morton *et al.*, 1993; Oda *et al.*, 1993), a subset of the breast cancer lines with altered expression of α- or β-catenin protein may have specific mutations in the corresponding gene. An alternative, but not mutually exclusive, explanation is that an assortment of alterations in post-transcriptional and post-translational regulation of catenin expression may account for the decreased levels of catenin proteins in the cancer cell lines.

While immunohistochemical studies suggest that ductal and lobular breast cancers have some differences in the prevalence of altered E-cadherin and α-catenin expression (Ochiai *et al.*, 1994), it was not clear a priori whether cell lines derived from breast cancers of differing histopathological types would display distinctly different patterns of expression. Eight of the cell lines in this study were reportedly derived from tumors with ductal histopathology (ATCC:BT-474, ZR-75-30, Hs578t, ZR-75-1, BT-483, MDA-MB-435s, T-47D, BT-549). Alterations in E-cadherin or catenin expression were seen in only a subset of these eight lines and were also seen in a similar percentage of the other lines for which the histology of the primary tumor was not noted. Thus, alterations in E-cadherin and catenin expression do not appear to be restricted to breast cancers of a particular histologic subtype.

The adhesive capacity of breast cancer-derived cell lines in Matrigel has been correlated with their expression of E-cadherin and vimentin (Sommers *et al.*, 1991; Thompson *et al.*, 1992). Data from those studies suggested E-cadherin expression correlated with the ability of a cell line to form spherical colonies or non-invasive clusters in Matrigel and that cell lines with high levels of vimentin expression formed invasive colonies. Nevertheless, these *in vitro* growth properties may not fully reflect appropriate function of the cadherin-catenin pathway, as one cell line (MDA-MB-468) that failed to express α-catenin protein and another cell line (SK-BR-3) lacking E-cadherin and β-catenin protein formed spherical colonies/non-invasive clusters in matrigel (Thompson *et al.*, 1992).

In summary, the data presented here suggest that alterations in E-cadherin and α- and β-catenin expression are common in breast cancer cell lines. Although we cannot exclude the possibility that alterations in E-cadherin and catenin expression and gene structure may have arisen during the establishment and subsequent passage of the cell lines, the prevalence of altered expression of E-cadherin and α-catenin in the breast cancer cell lines appears to be relatively well-correlated with the results obtained from previous immunohistochemical studies of primary breast cancers. Additional studies will be necessary to further elucidate what appears likely to be a complex assortment of mutational and altered regulatory mechanisms underlying the alterations in E-cadherin and catenin expression in breast cancer cells. Moreover, while these data provide further support for the proposal that defects in the E-cadherin-catenin pathway may be a critical and necessary step in the generation of advanced breast cancer cells, definitive functional studies will ultimately be required to establish the relationship and significance of our observations to the altered phenotypic properties of breast cancer cells observed *in vivo*.

Materials and methods

Cell lines

All cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in the recommended growth media. Cultures were incubated at 37°C with 5% CO_2, except for cultures maintained in Lebovitz's L-15 media which were grown at 37°C without CO_2. DNA, RNA, and protein were isolated from cultures at 75–95% confluence.

Western blot analysis

Cells were washed and then lysed in RIPA buffer [25 mM Tris-buffered saline (pH 8) with detergents (1% deoxycho-

late, 0.1% sodium dodecyl sulfate, 1% nonidet P-40) supplemented with 10 μg ml^{-1} phenylmethylsulfonyl fluoride (PMSF), 50 μg ml^{-1} antipain, 5 μg ml^{-1} aprotinin, and 2 μg ml^{-1} leupeptin (all protease inhibitors purchased from Sigma Chemical Co., St. Louis, MO). Protein concentrations were determined using the BCA Protein Assay Reagent (Pierce, Rockford, IL), using bovine serum albumin to generate a standard curve. Forty micrograms of total protein per sample was separated by electrophoresis on SDS/polyacrylamide gels and transferred to Immobilon P membranes (Millipore, Bedford, MA) by semi-dry electroblotting (Tansblot, Bio-Rad, Hercules, CA). Western blot analysis was carried out using affinity-purified polyclonal rabbit and rat or mouse monoclonal antisera as primary antibodies and horseradish peroxidase-conjugated goat anti-rabbit, goat-anti-rat or goat-anti-mouse immunoglobulin antibodies (Pierce) as secondary reagents. E-cadherin was detected with a rat monoclonal antibody DECMA-1 (Sigma). A polyclonal rabbit antiserum YR4 against α-catenin was generated by immunization with a bacterial recombinant protein containing the carboxy-terminal 447 amino acids of α-catenin fused to glutathione S-transferase (GST) (Rimm *et al.*, 1995). A rabbit polyclonal antisera against β-catenin was generated by immunization with a bacterial recombinant GST fusion protein containing full-length β-catenin sequence (D Rimm and E Koslov, unpublished observations). A polyclonal rabbit antiserum against βII-spectrin (β-fodrin) was generated by immunization with a bacterial recombinant GST fusion protein containing the carboxy-terminal third of human β-fodrin (SP Kennedy and JS Morrow, unpublished observations). The α-subunit of Na^+-K^+-adrenosine-triphosphatase (Na^+-K^+-ATPase) was detected using mouse monoclonal antibody C464.6 (a gift from Dr M Kashgarian, Dept. of Pathology, Yale University) which has been previously described (Kashgarian *et al.*, 1985). Band 4.1 protein was detected with a polyclonal rabbit antiserum raised against native band 4.1 protein purified from a red cell ghost lysate (Croall *et al.*, 1986). HER-2/Neu protein was detected by rabbit polyclonal antiserum Ab-1 (Oncogene Science, Uniondale, NY). Detection of antibody complexes was carried out with the Enhanced Chemiluminescence (ECL) Western Blot Kit (Amersham, Arlington Heights, IL) and subsequent exposure to Hyperfilm (Amersham). Western blot data shown in Figure 1 are representative of results obtained from studies performed two or more times.

Ribonuclease protection assay

Total RNA was isolated as described previously (Chomczynski and Sacchi, 1987) or using Trizol reagent (Gibco/BRL Life Technologies). Radiolabeled antisense riboprobe transcripts were prepared from plasmid constructs using T7 or T3 RNA polymerases (GibcoBRL/Life Technologies) or SP6 (New England Biolabs, Bedford, MA) and ^{32}P-CTP. Antisense riboprobes were generated from the following cDNA fragments: (i) *E-cadherin* – a 565 bp fragment containing 438 nucleotides of carboxyl-terminal coding sequences and 127 nucleotides of 3′ untranslated sequences; (ii) *α-catenin* – a 498 bp fragment corresponding to codons 449–615; (iii) *β-catenin* – a 635 bp fragment corresponding to codons 363–575; and (iv) *γ-actin* – a 275 bp fragment derived from the 3′ coding region of the cDNA. Transcripts were purified by electrophoresis, and 2.5×10^4 c.p.m. of each transcript was incubated overnight at 48°C with 5 μg of total RNA in hybridization solution [80% deionized formamide; 40 mM 1,4-piperazinediethane sulfonic acid (PIPES), pH 6.4; 400 mM NaCl; 1 mM EDTA]. The *γ-actin* transcript was co-incubated with the *E-cadherin*, *α-catenin* or *β-catenin* transcripts to control for RNA integrity and loading. Ribonuclease digestion was then carried out for 1 h at 30°C using 7.5 units of RNase T2 (Gibco BRL/Life Technologies, Grand Island, NY) in 250 μl of digestion buffer (50 mM sodium acetate, pH 4.4; 100 mM NaCl; 10 mM EDTA). RNA was precipitated with isopropanol, resuspended in RNA loading buffer (90% deionized formamide; 10 mM EDTA; 0.2% bromophenol blue; 0.2% xylene cyanol), heated for 3 min at 90°C and electrophoresed on a sequencing gel. After drying the gel, autoradiography was carried out with intensifying screens and Hyperfilm (Amersham) at –80°C. Data shown in Figure 2A–C are representative of results obtained in RNase protection assays performed two or more times.

Southern analysis

High molecular weight genomic DNA was isolated by incubation of cell pellets in 1.0% sodium dodecyl sulfate (SDS), 0.5 mg ml^{-1} proteinase K (Gibco BRL/Life Technologies) at 48°C for 24–48 h. After two extractions with phenol:chloroform:iso-amyl alcohol (50:49:1) and one extraction with chloroform:iso-amyl alcohol (49:1), DNA was recovered by ethanol precipitation. DNA concentrations were determined using a diphenylamine assay (Shatkin, 1969). For Southern analysis, 10 μg of genomic DNA was digested with EcoRI (Gibco BRL/Life Technologies), precipitated, and electrophoresed on 1.0% agarose gels. Transfer to Zeta-probe membranes (Bio-Rad) was performed using a positive pressure blotting apparatus (Posiblotter, Stratagene, San Diego, CA). Hybridization of the filters to *E-cadherin* and *α-catenin* cDNA fragments was carried out as described (Reale *et al.*, 1994). After post-hybridization washing, filters were exposed (tgo) Hyperfilm (Amersham) with intensifying screens at –80°C.

RT-PCR SSCP analysis

cDNA was prepared from DNase I-treated total RNA using random hexamers and Superscript reverse transcriptase (Gibco BRL/Life Technologies). *E-cadherin* sequences were then amplified by 35 cycles of polymerase chain reaction (PCR), with each cycle consisting of 30 s at 94°C, 45 s at 56°C, and 1 min at 72°C (last cycle 5 min). The PCR was carried out in the presence of 1.0 μCi [α-^{32}P]dCTP using standard reaction reagents and the following E-cadherin oligonucleotide pairs: pair 1 - ECAD131: 5′ - GAGAGAGGCCGCGTCCTGGGCA - 3′ and ECAD513: 5′ - CCAGGTTTTTAGGAAATGGGCC-3′; pair 2 - ECAD431: 5′-CCTCAGAAGACAGAAGAGA-GAC - 3′ and ECAD958: 5′ - CCTGTGTTCCTGTTAATG-GTG-3′; pair 3: ECAD822: 5′-ACCTCTGTGATGGAGG TCACAG and ECAD1118: 5′-GGGATTGAAGATCGGA GGATTATC-3′; pair 4: ECAD1003: 5′-CTACGTATACC-CTGGTGGTTCA-3′ and ECAD1365: 5′-CCACATTCGT-CACTGCTACG - 3′; pair 5: ECAD1473: 5′ - TCCGAGGA CTTTGGCGTGGGC-3′ and ECAD1790: 5′-GAATA-TAGTTCGAGGTTCTGGTAT-3′; pair 6: ECAD1731: 5′-CTGCTGATCCTGTCTGATGTG-3′ and ECAD2113: 5′-GCAGGAATTTGCAATCCTGCTTCG-3′; pair 7: ECAD 2080: 5′ - CACAGCCTGTCGAAGCAGGATTGC - 3′ and ECAD2524: 5′-CTCAGACTAGCAGCTTCGGAACCGC-T-3′; and pair 8: ECAD2490: 5′-TATGAAGGAAGCGG-TTCCGAA-3′ and ECAD2688: 5′-ACGCTGATTTCTG CATTTCTGCAC-3′. Following amplification, 1.5 μl from each 10 μl reaction was diluted into 3.5 μl formamide sequencing stop solution, heated to 90°C for 5 min, quickly chilled on ice, and then loaded immediately onto 5% Long Ranger (AT Biochem, Malvern, PA)/10% glycerol/0.6% TBE buffer sequencing type gels and electrophoresed at 15 W for 7 h at room temperature. The gel was dried and autoradiography was carried out at –80°C with intensifying screens and Hyperfilm (Amersham).

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