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Mechanisms of inactivation of E-cadherin in breast carcinoma: modification of the two-hit hypothesis of tumor suppressor gene

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Loss of heterozygosity (LOH) allows the expression of recessive mutation in tumor suppressor genes (TSG). Therefore, on the basis of Knudson's 'two-hit' hypothesis for TSG inactivation, the detection of a high LOH frequency in a chromosomal region is considered critical for TSG localization. One of these LOH regions in breast cancer is 16q22.1, which has been suggested to reflect the involvement of *E-cadherin* (*E-cad*), a cell–cell adhesion molecule. To confirm the tumorigenic role of *E-cad*, 81 sporadic invasive ductal carcinomas (IDCs) of the breast were tested for the 'two hits' required to inactivate this gene. A high frequency (37.3%) of LOH was detected in 67 informative tumors, but no mutation was found. To examine the possibility that transcriptional mechanisms serve as the second hit in tumors with LOH, specific pathways, including genetic variant and hypermethylation at the promoter region and abnormal expression of positive (WT1) and negative (*Snail*) transcription factors, were identified. Of these, promoter hypermethylation and increased expression of *Snail* were found to be common (>35%), and to be strongly associated with reduced/negative E-cad expression ($P<0.05$). However, unexpectedly, a significantly negative association was found between the existence of LOH and promoter hypermethylation ($P<0.05$), which contradicts the 'two-hit' model. Instead, since they coexisted in a high frequency of tumors, hypermethylation may work in concert with increased *Snail* to inactivate *E-cad* expression. Given that *E-cad* is involved in diverse mechanisms, loss of which is beneficial for tumors to invade but may also trigger apoptosis, this study suggests that maintaining a reversible mechanism, either by controlling the gene at the transcriptional level or by retaining an intact allele subsequent to LOH, might be important for *E-cad* in IDC and may also be common in TSGs possessing diverse functions. These findings provide clues to explain why certain TSGs identified by LOH cannot fulfil the two-hit hypothesis. *Oncogene* (2001) **20,** 3814–3823.

Keywords: E-cadherin; breast cancer; two-hit hypothesis; loss of heterozygosity; hypermethylation; snail

Introduction

Patients with a strong hereditary component account for a very small fraction of all cancer cases. However, the search for genes responsible for hereditary cancers is considered to be very important, not only because the study of family cancer genes can always provide critical insights into the origin and nature of tumorigenesis (Fearon, 1997), but also because, on the basis of the well-known two-hit inactivation model proposed by Knudson (1971), such genes often also play a role in the much more common sporadic form of the same tumor. Chromosomal loss of the wild-type allele always represents the most common of the two hits needed to inactivate tumor suppressor genes (TSGs), allowing the expression of recessive loss-of-function mutations in TSGs (Lasko *et al.*, 1991). The identification of a high frequency of genomic deletion detected by allelic loss or loss of heterozygosity (LOH) in specific genomic regions is therefore widely used in localizing sites of candidate TSGs. The two-hit model, implying germ-line mutation in one TSG allele accompanied by somatic loss (LOH) of the remaining wide-type allele, has held true for most TSGs in explaining cancer predisposition in family cancer syndromes. However, in sporadic cancers, with the exception of some TSGs, e.g. *p53*, *RB*, and *APC*, the probability of finding somatic mutation in certain TSGs with a high frequency of LOH has been shown to be extremely rare (Brown, 1997). Failure to detect mutation is usually explained by the presence of other undefined TSGs, that are located close to the original TSGs and are actually responsible for the observed LOH. However, recent evidence, which suggests two non-mutually exclusive possibilities, has provided clues in favor of a tumorigenic role of these TSGs defined

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solely by LOH in sporadic cancer. The first is that the TSG may exhibit the haplo-insufficiency phenotype, which shows that homozygous inactivating mutations and complete loss of function are not necessary to cause defective tumor suppressor function (Venkatachalam *et al*., 1998; Fero *et al*., 1998; Xu *et al*., 2000; MacLeod, 2000). Thus, a half-normal level of the gene product in sporadic cancer, resulting from LOH *per se*, would result in the phenotypic manifestation. The second possibility is that, in addition to somatic mutation, epigenetic mechanisms are involved in abrogating the function of these TSGs which are already targeted by LOH (Jones and Laird, 1999; MacLeod, 2000). The identification of numerous hypermethylated TSG promoters, together with a better understanding of gene-silencing mechanisms, has lent support to this second possibility (Baylin *et al*., 1998; Jones and Laird, 1999). For instance, *BRCA1*, which is defined as a TSG by the two-hit model in familial breast cancer syndromes, has been demonstrated to fit this 'LOH-plus-hypermethylation' model in sporadic breast cancers (Esteller *et al*., 2000). Consequently, these observations have led to the suggestion that Knudson's two-hit hypothesis could now be modified to include epigenetic mechanisms as one of the two hits required for TSG inactivation (Jones and Laird, 1999).

Recently, certain 'low-penetrant' alleles of a 'high-penetrant' TSG (e.g. *p53* and *BRCA2*), originally thought to confer an extremely high risk for the incidence of familial cancer syndromes, have been shown to play a role in sporadic cancer development (Storey *et al*., 1998; Healey *et al*., 2000). These polymorphic alleles would predispose carriers to a higher risk of developing cancer, but would not necessarily cause cancer. The probability of manifesting the tumorigenic phenotype depends on the interaction between these alleles and the environment, explaining why the increased cancer risk associated with variant alleles usually varies in different populations. The dependence of the manifestation of 'low-penetrant' alleles on the macroenvironment may also extend to the interaction with the cellular microenvironment. In contrast to the 'high-penetrant' tumorigenic effect of germ-line mutation of TSG in hereditary cancer, tumorigenic mechanisms linked to a defective TSG may manifest in a relatively mild ('low-penetrant') manner in sporadic cancer, and, accordingly, it is reasonable to speculate that mechanisms operating at the transcriptional level may play an important role in sporadic cancer development. This is mainly because, in contrast to genetic mutation, transcriptional mechanisms can switch genes on as well as off, affecting the level of expression of the normal TSG instead of totally abolishing TSG expression. Another important fact is that the reversibility of transcriptional mechanisms may provide an additional selective advantage for clonal evolution during tumorigenesis, since tumorigenesis is always characterized by an unstable, phenotypic heterogeneity which fluctuates too frequently to be mediated exclusively by rigid irreversible genetic changes (Graff *et al*., 2000). Promoter hypermethylation is one such reversible biochemical mechanism controlling genes at the transcriptional level and has been shown to play a role in tumor initiation, in particular, in sporadic cancer development.

In breast cancer, a high frequency of LOH is detected in the genomic region 16q22.1 (Lindblom *et al*., 1993; Shen *et al*., 2000). Of particular interest is the fact that, in our recent genome-wide study, LOH at this region was defined as an initiating step in tumor progression (Shen *et al*., 2000). It has been hypothesized that such LOH reflects the involvement of *E-cadherin* (*E-cad*) (Bièche and Lidereau, 1995; Ingvarsson, 1999), a cell-cell adhesion molecule implicated in metastasis suppression (Semb and Christofori, 1998; Guilford, 1999). Consistent with this hypothesis are the observations that, in breast cancer, deletion at 16q22.1 is associated with the development of distant metastasis (Lindblom *et al*., 1993) and reduced E-cad expression is associated with invasiveness and an unfavorable prognosis (Siitonen *et al*., 1996; Tan *et al*., 1999). Furthermore, loss of *E-cad*-mediated cell adhesion as a driving force in the progression from adenoma to carcinoma has been demonstrated in a transgenic mouse model of pancreatic β-cell carcinogenesis (Perl *et al*., 1998). To answer the questions whether *E-cad* is the target of LOH at 16q22.1 and whether *E-cad* is a TSG in breast cancer, we sought to identify the molecular mechanisms for the two hits inactivating this gene in sporadic infiltrating ductal carcinoma (IDC) of the breast. A range of *E-cad* alteration profiles, including genetic mutation, LOH, and promoter mutation and hypermethylation, was investigated in order to dissect out all possible genetic and epigenetic mechanisms. Furthermore, to assess a possible contribution of the cellular microenvironment, abnormal expression of positive and negative transcription regulators was examined. We hypothesized that, in sporadic cancer development, epigenetic mechanisms and the effects of the cellular microenvironment leading to reduced E-cad expression might play an important role. This study reports an investigation to test this hypothesis.

Results and discussion

To detect allelic loss of *E-cad*, three microsatellite markers (*D16S3095*, *D16S421*, and D16S752) at 16q22.1 (Figure 1a) were used. The allelic status of *E-cad* is well reflected by these three markers, as the *E-cad* locus is very close to the loci of these markers (lod score >4 in relation to *E-cad* estimated by linkage analysis), which have been used to define a causal role of *E-cad* in the development of hereditary cancer (Guilford *et al*., 1998). For LOH detection, the laser capture microdissection technique (Shen *et al*., 2000) was used to obtain pure tumor tissue, thus avoiding the problem of contamination with normal tissue. We considered the results for all three markers together and found that 67 (82.7%) cases showed heterozygous

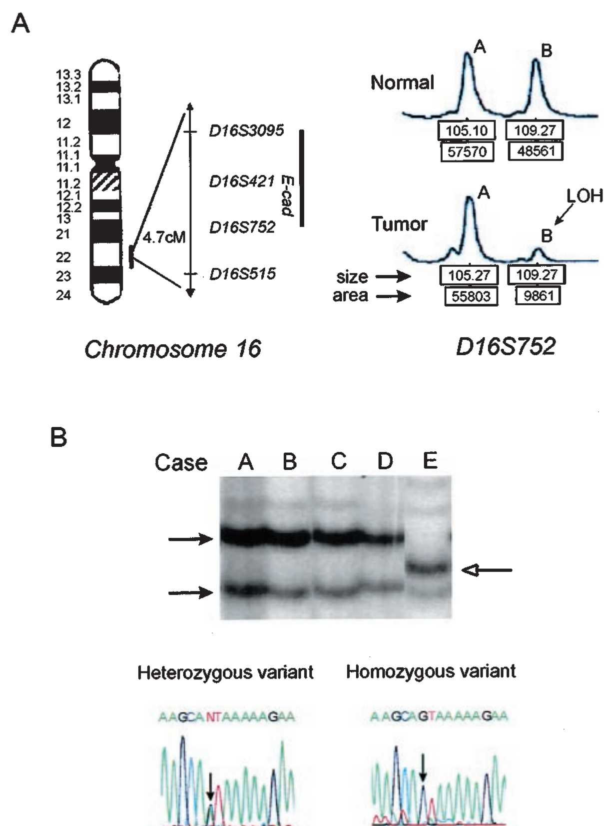

Figure 1 Genetic deletion and mutation of *E-cadherin (E-cad)*. (**a**) (Left panel) Deletion of *E-cad* detected by allelic loss or loss of heterozygosity (LOH) of the *E-cad* locus, reflected by three microsatellite markers (*D16S3095*, *D16S421*, and *D16S752*) at 16q22.1. For comparison, one marker (*D16S515*) located at 16q22.3 was also used. (Right panel) LOH in a representative IDC. The locus of marker *D16S752* was considered to be informative when, in normal tissue, it was heterozygous (i.e. two alleles, A and B, were seen), and shows LOH when a threefold or greater difference was seen in the relative allele intensity ratio (A : B) between the tumor and normal DNA (arrow). (**b**) *E-cad* mutation detected by PCR-single-stranded conformation polymorphism (PCR–SSCP) (upper panel) and direct DNA sequencing (lower panel). In SSCP, in contrast to the wild-type alleles (solid arrows), a band shift was seen in the Case E (open arrow), implying possible variation in DNA sequences. Two tumors showing band shifts in SSCP were subjected to DNA sequencing (lower panel), and were found to harbor heterozygous or homozygous A-to-G transitions in intron 12 of *E-cad*, respectively

in at least one of the three. A high frequency (37.3%) of allelic loss (Figure 1a) at *E-cad* was detected. This frequency is considered to be very high on the basis of the following comparative observations: (a) it is significantly higher than the LOH frequency (22.4%) detected at the marker representing the allelic status of an adjacent locus, 16q22.3 (Figure 1a) ($P<0.05$, *t*-test); (b) on the basis of an LOH frequency profile using 400 markers established by our recent genome-wide LOH study (Shen *et al*., 2000), the LOH frequency at the *E-cad* locus seen in the present study is among the top 10%; (c) assuming a normal distribution for the frequency of LOH at all genomic loci in our breast cancer patients, the frequency of LOH at 16q22.1 is among the top 15% for all loci. The 67 tumors that were informative for LOH status were subjected to further analysis in order to look for molecular mechanisms of inactivation of *E-cad*.

Prompted by the high LOH frequency at the *E-cad* locus, we screened the full coding sequence and splice junctions of *E-cad* for somatic mutations in our breast cancer patients. Although *E-cad* mutation is reported to be extremely rare in IDC (Berx *et al*., 1996), in order to address possible ethnic differences, which have been demonstrated in breast cancer in Chinese women harboring unexpected *BRCA1* somatic mutations (Khoo *et al*., 1999), the present study screened the entire *E-cad*. With two exceptions which showed the same nucleotide changes in intron 12 (Figure 1b), no somatic mutations were detected; moreover, the change at intron 12 did not affect *E-cad*, since no alternative splicing form was detected by RT–PCR. The absence of somatic mutation in our patients with IDC of the breast is consistent with previous results based on a smaller series of IDC patients (Berx *et al*., 1996). Interestingly, in contrast to the situation in IDC, a high frequency of *E-cad* mutation is found in the less common histological type of breast cancer, infiltrating lobular carcinoma (ILC) (Berx *et al*., 1995, 1996), in which more than 50% of tumors possess an *E-cad* mutation, and, in accordance with the two-hit hypothesis, the majority of mutations are found in combination with LOH of the wild-type *E-cad* locus. This result in ILC provides a biological basis to support a causal link between *E-cad* inactivation and breast cancer formation. Thus, in IDC, since the results show a high LOH frequency, but no somatic mutation, it is conceivable that other mechanisms for *E-cad* inactivation might exist.

To explain the high LOH frequency found at the *E-cad* locus in IDC and to determine whether *E-cad* was inactivated by mechanisms operating at the transcriptional level, we examined the involvement of two common mechanisms, promoter polymorphism and hypermethylation, which have been suggested to affect *E-cad* RNA expression. Recent observations identifying a C/A single nucleotide polymorphism at -160 from the transcriptional start site of the *E-cad* gene promoter (Figure 2a) show that the A allele of this polymorphism might alter transcription factor binding, resulting in a reduction in transcriptional efficiency of 68% compared with the C allele (Li *et al*., 2000). The PCR-based restriction fragment length polymorphism (RFLP) assay (Figure 2b) showed that 52.2% of our cases harbored at least one A (low-activity) allele. However, in contrast to genetic mechanisms (mutation and LOH), the effects of which are direct and fixed, mechanisms operating at the transcriptional level may vary between different cell types and depend on the interaction with the cellular microenvironment. More specifically, although the polymorphism at -160 is located within the region shown to possess promoter activity for *E-cad*, no known transcriptional factor binding sites show homology to the sequence around

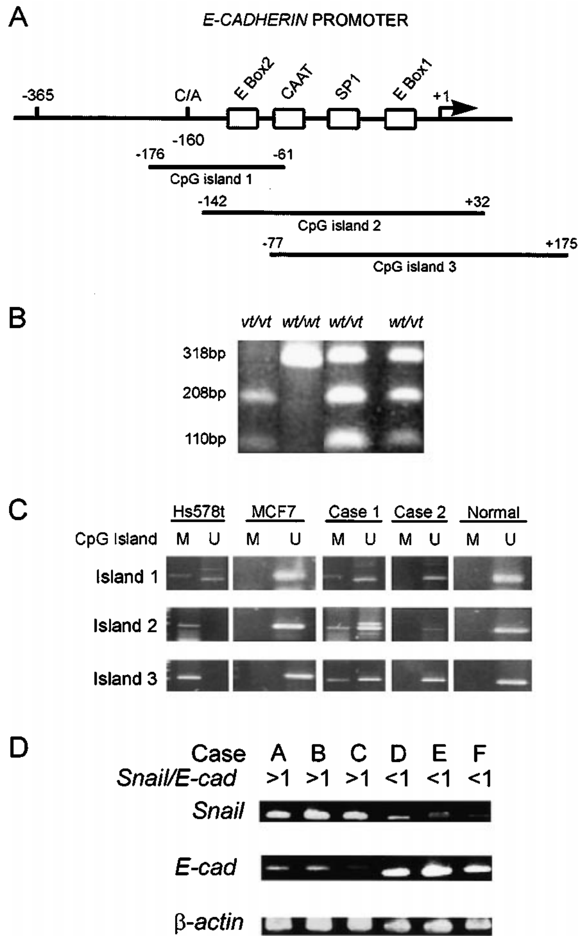

Figure 2 Transcriptional mechanisms suggested to be implicated in reduced/negative *E-cadherin (E-cad)* expression. (**a**) Schematic diagram of the *E-cad* promoter showing the −160 polymorphic site, the E-boxes for Snail binding, and three CpG islands implicated in hypermethylation. (**b**) PCR-restriction fragment length polymorphism (RFLP) analysis of genetic polymorphism of the −160 site of the *E-cad* promoter. The C/A polymorphism was differentiated by *Bst*II digestion of PCR products homozygous for the wild-type (high-activity) allele (*wt/wt*), heterozygous for the variant (low-activity) allele (*wt/vt*), and homozygous for the low-activity allele (*vt/vt*). (**c**) Promoter hypermethylation of the *E-cad* detected by methylation-specific PCR (MSP). The presence of a visible PCR product in the lanes marked U indicates the presence of an unmethylated allele, while the presence of product in the lanes marked M indicates the presence of a methylated allele. The cell line Hs578t, used as the positive control, shows heterogeneous methylation in CpG island 1 and complete methylation in islands 2 and 3, and the cell line MCF7 was used as the negative control (Graff *et al*., 1997). One tumor (Case 1) harbored a hypermethylated allele, and the other (Case 2) did not show evidence of promoter hypermethylation. Normal breast tissue (Normal) did not contain hypermethylated *E-cad*. (**d**) RT–PCR detection of relative expression of *Snail* and *E-cad*. RT–PCR using primer pairs specific for *Snail* and *E-cad* was performed on individual tumors (Cases A–F) and an inverse relation was seen between the expression of *Snail* and *E-cad*, increased *Snail* expression being defined by a *Snail*/*E-cad* ratio >1. The expression of β-actin was analysed in the same tumors as a control for the amount of RNA (cDNA) present in each sample

the C/A polymorphism site (Li *et al*., 2000). We therefore first checked whether this polymorphism in the *E-cad* promoter was associated with E-cad expression in our tumors. To avoid the problem of ambiguous and objective definition of level of gene expression, which appears to be common when observing heterogeneous cell populations such as primary tissue, we used a combination of immunoblotting (IB) and immunohistochemistry (IHC) to determine the level of *E-cad* expression (Figure 3). The protein expression level was considered to be the endpoint of interest, since it is of clinical significance because of the association between poor progression of breast cancer and reduced E-cad expression (Siitonen *et al*., 1996; Tan *et al*., 1999). With the exception of two tumors which were positive by IB but showed mislocalization of E-cad to the cytoplasm detected by IHC (Figure 3), consistent findings of E-cad expression (positive vs. reduced/negative expression) were obtained using the two different methods. About 50% of the 67 breast tumors showed reduced or no expression of E-cad (Table 1), a figure similar to that reported by IHC alone. We found no significant correlation between expression level and genotype polymorphism of the *E-cad* promoter (Table 1). In addition, the *E-cad* LOH of tumors did not correlate with the allelic/genotypic status of promoter polymorphism ($P>0.20$). Since a gene-dose effect between promoter polymorphism and phenotypic manifestation of E-cad has not yet been demonstrated *in vivo*, we tried different grouping strategies to define the genotype and expression status, but without success. Accordingly, these findings do not confirm reduced expression of E-cad conferred by the A allele in primary breast cancer tissue and argue against the hypothesis that genotype polymorphism at the *E-cad* promoter accompanying genetic deletion (LOH) serves as the second hit in the inactivation of *E-cad* in IDC.

E-cad is one of the TSGs in which aberrant DNA methylation of promoter region CpG islands (Figure 2a) serves as an alternative mechanism to coding region mutation for gene inactivation (Graff *et al*., 1997; Baylin *et al*., 1998; Fearon, 2000). Furthermore, methylation of the *E-cad* promoter has been documented as the second hit responsible for the development of hereditary gastric cancer in patients with one germ-line *E-cad* mutated allele (Grady *et al*., 2000). To obtain a comprehensive insight into the methylation pattern of the *E-cad* CpG island region in IDC, we used methylation-specific PCR (MSP) (Figure 2a) to study the methylation status of all three CpG islands located in the *E-cad* promoter. A high proportion (37.3%) of hypermethylated alleles were present in our breast carcinomas and all three islands were simultaneously hypermethylated in all hypermethylated tumors. To further explore whether there was variation in methylation status at different CpG sites in the same CpG island (as seen in hypermethylated *p16*, Gonzalgo *et al*., 1997; Woodcock *et al*., 1999), the methylation pattern of individual CpG sites was examined in greater detail by bisulfite-modified genomic sequencing (Figure 4) in selected tumor tissues, and a uniform pattern of methylation of entire CpG sites was

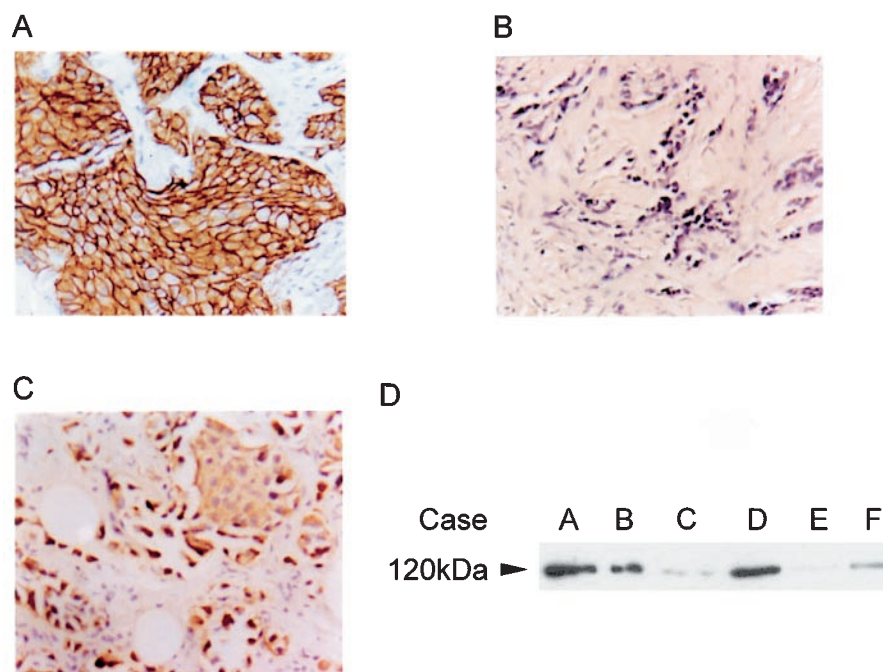

Figure 3 Detection of E-cadherin (E-cad) protein expression by immunohistochemistry (**a**, **b**, and **c**) and immunoblotting (**d**). (**a**) Tumor showing positive E-cad expression, in which more than 75% of the tumor cells gave a signal. (**b**) Tumor showing negative E-cad expression. (**c**) Tumor showing cytoplasmic staining for E-cad. (**d**). IB was used to obtain a semi-quantitative measurement of E-cad expression in tumors. E-cad expression was considered to be positive in cases A, B, and D, reduced in cases C and F, and negative in case E

Table 1 Transcriptional mechanisms in relation to E-cadherin (E-cad) expression in sporadic infiltrating ductal carcinoma of the breast[a]

	No. with reduced or no E-cad *(%)*	*No. positive for* E-cad *(%)*	P *value*[b]
Genotype of promoter polymorphism at −160			
vt/vt, vt/wt	19 (59.4)	16 (45.7)	0.26
wt/wt	13 (40.6)	19 (54.3)	
Promoter hypermethylation			
Yes	17 (53.1)	8 (22.8)	0.011
No	15 (46.9)	27 (77.2)	
Increased expression of *Snail*[c]			
Yes	29 (90.6)	1 (5.3)	0.0023
No	3 (9.4)	18 (94.7)	

[a]E-cad expression in breast tumors was detected and measured by both immunohistochemistry and immunoblotting. [b]*P* value determined by Chi-square test. [c]*Snail* detection was not performed on 16 tumors due to insufficient amounts of RNA

observed. A current model based on cell-line studies suggests that methylation is progressive, initially involving the CpG islands at the 5′-boundary of the promoter, gradually encompassing all the CpG islands of the promoter during tumor progression (Baylin *et al*., 1998). Thus, our result of concurrent methylation at all three CpG islands provides a clue that promoter hypermethylation during IDC tumorigenesis might occur at a very early stage, probably preceding the stage of tumor invasion (i.e. ductal carcinoma *in situ*, DCIS). Interestingly, our prediction based on molecular observation is consistent with that reported in a recent clinical study of ductal breast carcinoma, in which hypermethylation of the *E-cad* promoter was also reported as an early event, occurring prior to invasion in about 30% of DCIS lesions and increasing significantly to nearly 60% in metastatic lesions (Nass *et al*., 2000). The presence of promoter hypermethylation was considered to be functionally meaningful, as it was much more frequent in tumors with reduced or negative E-cad expression than in tumors showing positive E-cad expression (Table 1). This association justified further examination of the two-hit hypothesis that *E-cad* promoter hypermethylation is associated with the frequent loss of chromosomal material at one allele of *E-cad*, as seen in *BRCA1* (Esteller *et al*., 2000). We thus examined the hypermethylated status of tumors with or without LOH at the *E-cad* locus and found a statistically significant association ($P = 0.001$). Surprisingly, the result was the opposite of that predicted by the two-hit hypothesis, since hypermethylated alleles were much more common (50%) in the 42

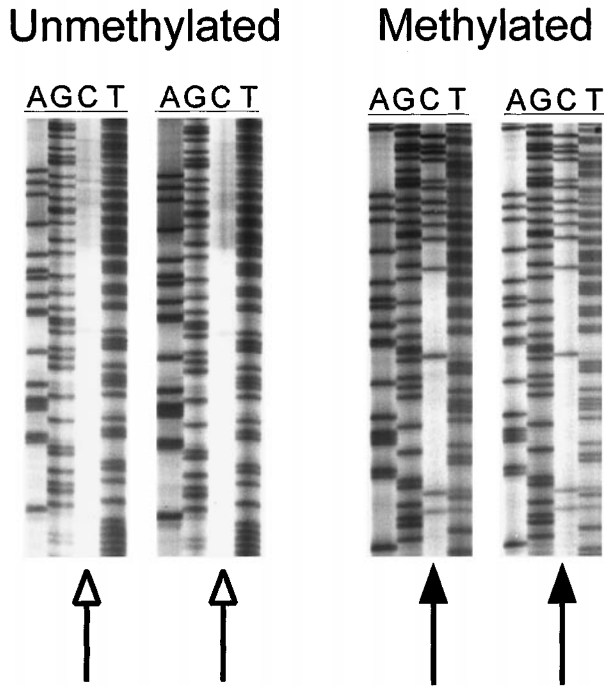

Figure 4 Bisulfite genomic sequencing to detect possible variation of methylation status at individual CpG sites of the *E-cadherin (E-cad)* promoter. In tumors harboring methylated alleles, the cytosine, locating in CpG island 3 of the *E-cad* promoter, is unchanged after sodium bisulfite treatment (solid arrows, right panel). In contrast, in tumors harboring unmethylated alleles, the cytosine is converted to thymine (open arrows, left panel)

tumors without *E-cad* LOH than in the 25 harboring LOH (20%) ($P<0.05$). Further examination using individual markers to re-define the LOH status of tumors yielded similar results. Taken together, our results define aberrant hypermethylation of the *E-cad* promoter as one of the important mechanisms contributing to reduced/negative E-cad expression in IDC. However, in contrast to the current prediction of the two-hit model, hypermethylation and LOH did not seem to occur in the same set of tumors. An inverse correlation between hypermethylation and LOH has also been seen in the inactivation of *MLH1* in colorectal cancers presenting microsatellite instability (Kuismanen *et al*., 2000). However, the mechanism is different in the case of *MLH1*, in which hypermethylation and LOH compete with each other to be the second hit. Thus, hereditary non-polyposis colorectal cancers carrying inherited *MLH1* mutations as the first hit did not show any promoter hypermethylation if LOH was present (Kuismanen *et al*., 2000). In contrast, in the case of *E-cad* in sporadic IDC, no somatic mutation was detected, and both hypermethylation and LOH occurred early at the same stage of tumor progression, suggesting that both served independently as the first hit and had similar functions in *E-cad* inactivation. Consequently, it is conceivable that the IDC in which one *E-cad* allele is lost and the IDC containing hypermethylated *E-cad* allele is present develop through two different tumorigenic pathways. In the present study based on primary tumor tissue, however, we were unable to delineate in tumors without LOH whether this epigenetic change was involving one or both alleles.

Because of these unexpected findings, since all known mechanisms that could possibly affect the gene itself had already been examined, we studied possible contributions from the cellular microenvironment which might be able to act in concert with the LOH or promoter hypermethylation implicated in *E-cad* inactivation. A common transcriptional mechanism known to affect TSGs is aberrant RNA splicing, resulting from an abnormal cellular microenvironment linked to overexpression of the protein (the SR protein family) regulating pre-mRNA splicing (Valcárcel and Green, 1996); however, this mechanism was not involved, since no abnormal *E-cad* transcripts were detected in this study. However, regulation of E-cad expression by the cellular microenvironment has been clearly demonstrated using somatic cell hybrids between breast cancer lines with intact E-cad transcription and lines lacking *E-cad* transcription that failed to express *E-cad* transcripts and protein, despite the fact that *E-cad* alleles from the intact *E-cad* line were present (Hajra *et al*., 1999). Defects in *trans*-acting pathways regulating E-cad expression is one explanation for the loss of E-cad expression in breast cancers (Ji *et al*., 1997). To address this possibility in primary tissues, we examined the contribution of suggested positive and negative transcription factors affecting E-cad expression. The transcription factor, Snail, has been recently demonstrated to bind to the *E-cad* promoter (E-box, Figure 2a) and to act as a repressor of E-cad expression in epithelial tumor cell lines (Batlle *et al*., 2000; Cano *et al*., 2000). Using RT–PCR, we measured the relative expression of *Snail* and *E-cad* in our breast tumors (Figure 2d), increased *Snail* expression being indicated by a value for the *Snail*/*E-cad* RNA expression ratio >1. It was interesting to find that almost all tumors displaying reduced/negative E-cad expression showed increased *Snail* expression, while, in sharp contrast, only 5.3% of tumors with positive E-cad expression showed increased *Snail* expression (Table 1). One cautionary note should be raised that this association was identified by RT–PCR, and further study using simultaneous immunohistochemistry to detect Snail and E-cad expression in the same tumor cells would be very helpful to confirm this possibility. Using a similar approach, we examined the contribution of the positive transcription factor, WT1, which binds to the promoter (GC-rich sequences) of *E-cad*, resulting in increased expression of *E-cad* mRNA (Hosono *et al*., 2000). Unfortunately, almost all our IDC tumors (87%) showed undetectable levels of *WT1* mRNA, a figure consistent with that reported previously (Silberstein *et al*., 1997; Laux *et al*., 1999; Fabre *et al*., 1999), this being a result of promoter hypermethylation and genetic deletion of the locus of *WT1* (11p13). In these *WT1*-negative tumors, a significant proportion (37.1%) still expressed E-cad.

These findings therefore suggest that the loss of the tumor suppressor, *WT1*, might contribute significantly to breast tumorigenesis, but that WT1 does not play an important role in regulating E-cad expression in IDC.

We next examined whether increased expression of the negative regulator, Snail, could replace promoter hypermethylation as the transcriptional mechanism which, together with the loss (LOH) of *E-cad*, provided the two hits inactivating *E-cad* in IDC. However, this possibility could be excluded, since no association was seen between *E-cad* LOH and increased *Snail* expression, increased *Snail* expression being seen in 64% of tumors without LOH and 52% of tumors with LOH ($P=0.41$). These findings suggest that IDC already possessing one deleted *E-cad* allele do not show a higher tendency to recruit transcriptional mechanisms to inactivate the remaining intact allele. Consequently, it is reasonable to speculate that, for IDC development, there might be a selective growth advantage in retaining one intact *E-cad* allele rather than losing both alleles.

Because the binding site of Snail in the *E-cad* promoter is in three E-boxes, which are located within the CpG islands (Figure 2a), we investigated the possibility of a synergistic effect of increased expression of *Snail* and promoter hypermethylation. Interestingly, the possibility of these two transcriptional pathways working together is supported by a strong positive association between promoter hypermethylation and increased *Snail* expression, since 75% of 24 tumors harboring hypermethylated allele showed increased expression of *Snail*, while only 44.4% of 27 tumors without promoter hypermethylation showed increased *Snail* expression ($P=0.027$). Since the present study was carried out on primary tumor tissue, there was a possibility that intratumor heterogeneity, i.e. the tumor cells harboring hypermethylated allele and those showing increased *Snail* expression were from different clones and were actually in different cell populations, might result in a false positive correlation. To examine this possibility, we compared the proportion of tumors with reduced/negative E-cad expression in tumors with different statuses of *Snail* expression and promoter hypermethylation. Support for this putative interaction comes from the significantly higher proportion of tumors with reduced or negative E-cad expression in tumors with two transcriptional hits (94.4%, 17/18) compared with tumors with one hit (77.8%, 14/18) or in those harboring neither hypermethylated allele nor increased *Snail* expression (7%, 1/15) ($P<0.05$, Mantel-extension test for trend). Although the molecular mechanism remains to be explored, this strong association suggests that these two mechanisms operating at the transcriptional level exert a cooperative effect in reducing E-cad expression in IDC. A possible interaction between Snail and the methyl-CpG-binding proteins (e.g. MeCP2) will be an interesting topic for future study.

This study is the first to comprehensively investigate the possible molecular mechanisms involved in reduced E-cad expression in IDC. It is still possible that E-cad expression could be affected by other epigenetic mechanisms affecting E-cad itself (e.g. aberrant phosphorylation of E-cad) or by disruption of the interaction between E-cad and other partners (i.e. α,β,γ catenins and p120) involved in the same molecular pathways (Guilford, 1999). However, in contrast to the direct effect on E-cad expression caused by LOH and promoter hypermethylation detected in the present study, possible effects due to these undefined mechanisms are indirect and not fixed, since they are linked to translational or post-translational events, and, in addition, their contribution to breast cancer has never been reported. Taken together, our data suggest that: (i) genetic deletion of *E-cad*, detected by LOH, occurs in a significant proportion of IDC. However, in these tumors, the presence of LOH was the only event that could affect the function of *E-cad* directly and genetically, since no somatic mutation was detected. A revised two-hit model suggests that LOH would work in concert with transcriptional silencing mechanisms to inactivate the two alleles of a TSG. This hypothesis is supported by the observation that silencing of TSGs by promoter hypermethylation occurs more frequently in tumors in the presence of LOH, and, in sporadic breast carcinomas, *BRCA1* has been shown to follow this model (Esteller *et al*., 2000). However, in the present study, tumors in which *E-cad* had been deleted did not show an increased proportion of a second hit caused by transcriptional mechanisms, including promoter polymorphism; in fact, tumors with *E-cad* LOH appeared to purposely retain the remaining intact allele, as shown by a significantly lower proportion of promoter hypermethylation in these tumors, (ii) promoter hypermethylation and increased expression of the negative regulator, Snail, are dominant mechanisms affecting *E-cad* expression in IDC, which is consistent with our hypothesis that, in sporadic cancer, transcriptional mechanisms play an important role in inactivating TSGs. Moreover, these two mechanisms would work together to reduce E-cad expression in IDC. This observation not only could expand the current two-hit model to include the possibility that two transcriptional pathways make up the two hits, but also provides support for the presence of an amplified effect to inactivate *E-cad* by an aberrant allele containing hypermethylated promoter in a particular cellular microenvironment due to *trans*-acting effect of Snail. These findings, together with the recent unexpected observation of re-expression of E-cad in breast tumor cells in metastatic lesions (Ilyas, 2000; Bukholm *et al*., 2000), prompt us to propose that down-regulation of E-cad expression in IDC is transient in nature, and that complete or irreversible loss of *E-cad* may not confer a more selective advantage for breast cancer cells to progress. A more flexible status, either by retaining an intact allele subsequent to LOH or by regulation via epigenetic mechanisms operating at the transcriptional level, could provide an advantage in counteracting the changing microenvironment during tumor progression. This inference can be supported by recent findings showing the tumor microenvironment would simulta-

neously alter promoter methylation status and *E-cad* expression in a cell culture model (Graff *et al*., 2000), which further suggests the importance of remaining flexibility of cancer-associating genes to interact with the microenvironment. This flexibility may also reflect the diverse, and seemingly paradoxical, role of a multifunctional gene, such as *E-cad*, in tumor progression. On the one hand, *E-cad* inactivation results in alteration of cellular morphology (Christofori and Semb, 1999; Handschuh *et al*., 1999; Guilford, 1999), leading to reduced cellular adhesion and increased cellular motility which are of benefit in tumor invasion and metastasis; while, on the other, *E-cad* is also involved in maintaining cellular architecture and signaling microtubule stabilization (Christofori and Semb, 1999; Guilford, 1999; Chausovsky *et al*., 2000), which are essential for the survival of cells, even tumor cells. In addition, under specific circumstances, *E-cad* is able to conversely promote the expansion of intraepithelial neoplasia (Bindels *et al*., 2000). Thus, the loss of E-cad-mediated cell adhesion would lead to tumor cell apoptosis (Kantak and Kramer, 1998; Day *et al*., 1999; Ilyas, 2000). Two specific pathological manifestations seen in IDC provide additional evidence in favor of the idea of *E-cad* maintaining an intact allele or being regulated by epigenetic mechanisms: (a) most IDCs often infiltrate as cohesive groups of tumor cells, in contrast to the single cells seen in ILC (Berx *et al*., 1995), and (b) on IHC analysis, partial loss and variation in E-cad staining is commonly found. More importantly, heterogeneous methylation of the CpG islands in the *E-cad* promoter in IDC has been seen in a single tumor (Nass *et al*., 2000), different cells of which concurrently harbored either methylated or unmethylated alleles. The heterogeneity of transcriptional mechanisms parallels the changing and diverse role of *E-cad* during tumor progression. Given these observations, complete and permanent loss of E-cad expression is not required for a tumorigenic and invasive phenotype of sporadic IDC. Accordingly, we suggest that it is critical and beneficial for *E-cad* and other TSGs participating in multiple and diverse regulation pathways to maintain a dynamic status by the use of flexible mechanisms to temporarily silence or reactivate its function during clonal evolution. This specific arrangement involved in TSG inactivation may provide clues explaining why the somatic mutation rate is extremely low in certain TSGs already targeted by LOH and why certain TSGs identified by a high LOH frequency cannot fulfil the two-hit hypothesis.

Materials and methods

Study population, tumor tissue, and cell line

The study is part of an ongoing cooperative study aimed at understanding the causes of breast cancer in Taiwan, which is characterized by low incidence (Yang *et al*., 1997), early tumor onset (Lo *et al*., 1998), reproductive hormone-dependency (Huang *et al*., 1999), and novel genomic alterations (Lou *et al*., 1997; Lo *et al*., 1998). The study subjects (81 patients) were a subset of women randomly selected from this large, ongoing hospital-based breast cancer cohort. Their ages ranged from 40 to 85 years. None had a family history of breast cancer (mother or sisters). The tumors were diagnosed as histologically confirmed invasive ductal carcinomas. Institution review board-approved informed consent was obtained from each patient prior to tissue collection. None of the patients were receiving neoadjuvant treatment at the time of primary surgery. Tumor tissues were flash-frozen in liquid nitrogen and stored at $-80^{\circ}C$ until analysis. Peripheral blood was collected from each patient on the day of surgery and white blood cells were isolated by centrifugation. Genomic DNA, RNA, and protein were extracted from tumor specimens and DNA was extracted from blood cells by conventional protocols established in our laboratory (Tseng *et al*., 1997, Ku *et al*., 1997, Ma *et al*., 2000). Two human breast cancer cell lines (Hs578t and MCF7) were obtained to act as positive and negative controls for methylation-specific PCR (described below), and were cultured in modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 IU/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM) in a 37°C humidified incubator with 5% CO_2.

Allelotyping PCR and detection of allelic loss (LOH) of E-cad

In the search for possible mechanisms that act in concert with allelic loss (LOH) of *E-cad*, the present study began by defining the allelic status of *E-cad* in the tumors. To ensure that the tissue samples assayed consisted of $>90\%$ tumor cells, laser capture microdissection (LCM) was performed on routinely immunostained slides using a PixCell laser capture microscope (Arcturus Engineering, Mountain View, CA, USA) as described previously (Shen *et al*., 2000). DNA from the microdissected tumor specimens and blood samples was used for allelotyping PCR using fluorescent primers (markers). Three microsatellite markers (*D16S3095*, *D16S421*, and *D16S752*) at 16q22.1 were used to detect LOH at the *E-cad* locus; another marker (*D16S515*), located at 16q22.3, was used for comparison. PCR amplification was carried out following the previously described protocol (Shen *et al*., 2000). PCR products were electrophoresed on a 377 ABI PRISM sequencer, and the fluorescent signals from the different sized alleles were recorded and analysed using GENOTYPER version 2.1 and GENESCAN version 3.1 softwares. A given informative marker was considered to display LOH when a threefold or greater difference was seen in the relative allele intensity between tumor and normal DNA. However, because we used LCM in this study, almost all LOH markers showed a >fivefold difference in intensity.

PCR and single-stranded conformation polymorphism (PCR–SSCP), and DNA sequencing for E-cad *mutation analysis*

We used PCR–SSCP to determine inactivating mutations responsible for the loss of E-Cad expression. The promoter region and the 16 exons were analysed using the previously described protocol and primer pairs (Berx *et al*., 1995, 1996). All variants, i.e. DNA fragments showing mobility shifts, were re-amplified and the site of variation was identified by direct DNA sequencing using an ABI 377 autosequencer.

Restriction fragment length polymorphism (RFLP) to identify the nucleotide change at −160 of the E-cad *promoter*

The −160 polymorphic site contained either an A or a C allele. Tumor genotype was determined by *Bst*EII digestion

of the PCR products, amplified using the primer set of 5′-TGATCCCAGGTCTTAGTGAG-3′ (upstream) and 5′-AGTCTGAACTGAC TTCCGCA-3′ (downstream). The 318 bp PCR product was cut into two fragments (208 and 110 bp) if it contained the C allele. To ensure that the observed polymorphism was specific and was not due to experimental variation, the results were confirmed by direct DNA sequencing.

Methylation-specific PCR (MSP) and bisulfite-modified genomic sequencing to detect promoter hypermethylation of E-cad

Genomic DNA was modified by bisulfite treatment, converting unmethylated cytosines to uracils and leaving methylated cytosines unchanged (Herman *et al*., 1996). MSP was performed on this treated DNA to detect all three CpG islands of the *E-cad* promoter region (Figure 2a). Each unmethylated/methylated primer pair set was engineered to assess the methylation status of 4–6 CpGs with at least one CpG dinucleotide positioned at the 3′ end of each primer in order to facilitate discrimination between methylated and unmethylated alleles following bisulfite modification (Graff *et al*., 1997). The Hs578t cell, which contains a heterogeneously methylated CpG island 1 and methylated CpG islands 2 and 3 (Graff *et al*., 1997), served as the positive control, whereas the MCF7 cell acted as the negative control. In addition, to obtain a comprehensive picture of the methylation status of all CpG sites, PCR amplification of bisulfite-converted genomic DNA was performed under the conditions described previously (Gonzalgo *et al*., 1997). The PCR products were then sequenced using the Sequenase version 2.0 kit (Amersham, Cleveland, OH, USA).

RT–PCR to detect mRNA for E-cad, Snail, *and* WT1

Total RNA isolated from tumor tissues was reverse transcribed. Amplification of cDNA was performed using primers specific for *E-cad*, *Snail*, *WT1*, and β-actin (internal control). The primer sequences were: *E-cad*, (upstream) 5′-ACGATGATGTGAACACCTACA-3′ and (downstream) 5′-ATGCCATCGTTGTTCACTGCA-3′; *Snail*, (upstream) 5′-AATCGGAAGCCTAACTACAAG-3′ and (downstream) 5′-AGGAAGAGACTGAAGTAGAG-3′; *WT1*, (upstream) 5′-ATTCAGGATGTGCGACGTGT-3′ and (downstream) 5′-TGACAACTTGGCCACCGACA-3′; β-actin, (upstream) 5′-ACACTGTGCCCATCTACGAGG-3′ and (downstream) 5′-AGGGGCCGGACTCGTCATACT-3′. The annealing temperatures for individual primer sets and the number of reaction cycles in PCR were optimized to yield the greatest amount. The level of expression of a given gene in individual tumors was expressed as the ratio of the expression of the gene relative to that of β-actin.

Immunohistochemistry and immunoblotting to measure E-cad expression in tumors

In immunohistochemical studies, sections (5 μm thick) from fixed, paraffin-embedded tumors were reacted with monoclonal anti-E-cad antibody (Cappel, Aurora, OH, USA), followed by a second antibody, and the signal detected using the avidin-biotin complex system and diaminobenzidine (DAB) kit (Vector laboratories, Burlingame, CA, USA). DAB yielded a reddish brown stain if the sample was positive. ‘Positive staining’ for E-cad was assessed microscopically as a tumor in which 75% or more of the cells were stained. Positively stained cells were further examined to locate the cellular localization (nucleus or cytoplasm) of the signal.

To more precisely quantify the level of expression of E-cad in tumors, immunoblotting was performed following a protocol established in our laboratory (Ma *et al*., 2000). Membrane blots containing 40 μg of protein extracted from tumors were incubated with specific E-cad antibody (Zymed, San Francisco, CA, USA), followed by second antibody. Specific antibody-binding bands were revealed using horseradish peroxidase-conjugated anti-mouse IgG, and the intensity of the bands was scored as described previously (Davidson *et al*., 2000) and was classified as ‘positive’, ‘reduced’ and ‘negative’ E-cad expression.

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