BRCA1 and E-Cadherin Promoter Hypermethylation and Gene Inactivation in Cancer—Association or Mechanism?

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The abnormal properties of cancer cells are attributable to alterations in gene sequence and expression. The genes that are mutated in cancer can be grouped into two classes: proto-oncogenes and tumor suppressor genes. Proto-oncogenes are affected by gain-of-function mutations in cancer, generating oncogenic variant copies (or alleles) with increased or novel functions, while tumor suppressor genes are inactivated. Tumor suppressor genes were initially hypothesized to be inactivated in cancer cells as a result of genetic defects of both alleles (i.e., the Knudson two-hit hypothesis). Many studies have validated this concept, demonstrating localized mutations in both tumor suppressor gene alleles or a localized mutation in one allele coupled with a loss of heterozygosity (LOH) in the other allele. However, there is now evidence that epigenetic events, such as hypermethylation of cytosine–guanine (CpG) sites in regulatory regions (e.g., the promoter), may be a critical alternative mechanism of tumor suppressor gene inactivation, including von Hippel–Lindau inactivation in some clear-cell renal cancers, $p16^{INK4a}$ inactivation in some lung and other cancers, and MLH1 inactivation in many sporadic colon cancers with microsatellite instability *(1–3)*. Two reports *(4,5)* in this issue of the Journal provide additional data consistent with the view that hypermethylation of the promoter regions of certain tumor suppressor genes may play an important role in extinguishing gene expression in cancer. However, before accepting the conclusion that hypermethylation of tumor suppressor gene promoters is invariably the cause of gene inactivation, it is worth evaluating the data in the two reports a bit more critically.

Perhaps the first clear evidence that a single gene might underlie breast cancer risk in some families was provided by the mapping of BRCA1 (breast cancer predisposition gene 1) at chromosome 17q21 *(6)*. Further work *(7,8)* revealed that germline BRCA1 mutations also substantially increase ovarian cancer risk, and the BRCA1 gene was ultimately identified in 1994 *(9,10)*. BRCA1 germline mutations may underlie cancer risk in about half of families with four or more cases of breast cancer diagnosed before age 60 years and three quarters of families with both breast and ovarian cancers *(11)*. On the basis of the fact that LOH at the BRCA1 locus was seen in roughly 50% of unselected breast cancers and in 60%–80% of unselected ovarian cancers *(11,12)*, BRCA1 inactivation was initially hypothesized to have an important role in sporadic cancers. Surprisingly, somatic BRCA1 mutations in sporadic breast carcinomas have not been described *(11)*, and somatic BRCA1 mutations have been identified only rarely in sporadic ovarian carcinomas *(11,13)*. Nevertheless, prior work has provided evidence that BRCA1 may be inactivated in some nonfamilial cancers by mechanisms other than coding region mutations. Loss of BRCA1 transcripts has been observed in some nonfamilial breast cancers *(14–16)*, and a recent comprehensive immunohistochemical study *(17)* indicated that, while BRCA1 protein expression was uniformly present in normal breast tissues, lobular carcinomas, and low-grade ductal carcinomas, the majority of high-grade ductal carcinomas had reduced or undetectable BRCA1 expression. Some ovarian carcinomas also demonstrated loss of BRCA1 expression *(17)*. Increased methylation of CpG sites in the BRCA1 promoter has previously been proposed to underlie BRCA1 inactivation in some cases *(15,16,18–20)*.

In the first report presented in this issue of the Journal, Esteller et al. *(4)* have extended studies of BRCA1 promoter methylation in breast and ovarian cancers. A principal finding of their work was that the BRCA1 promoter was unmethylated in normal tissues and in all breast cancer cell lines tested, but BRCA1 promoter hypermethylation was present in 11 (13%) of 84 unselected breast carcinomas. BRCA1 hypermethylation was most common in lesions with medullary and mucinous differentiation, two histologic types that are more common in breast carcinomas arising in individuals carrying a germline BRCA1 mutation than in unselected cases *(21)*. Studies of the relationship between LOH at the BRCA1 locus and BRCA1 promoter hypermethylation indicated that nine (20%) of 45 tumors with LOH had BRCA1 hypermethylation, while one (5%) of 21 without LOH was methylated. The authors also found that BRCA1 hypermethylation was tightly associated with LOH at the BRCA1 locus in ovarian cancer. Finally, although the authors only studied the relationship between BRCA1 methylation and gene expression in six breast cancer xenografts (two of which showed BRCA1 hypermethylation and undetectable BRCA1 transcripts), they concluded that silencing of BRCA1 occurs by promoter hypermethylation in primary breast and ovarian carcinomas.

The conclusions by Esteller et al. *(4)* regarding a cause-and-effect relationship between BRCA1 promoter hypermethylation and inactivation may ultimately be well established. Nevertheless, uncertainties remain at this time. While the authors did not study BRCA1 expression in their primary tumor specimens, their claim that 13% of unselected breast cancers and 13% of ovarian cancers are likely to lack BRCA1 expression as a result of BRCA1 hypermethylation is not inconsistent with prior results *(14–17)*. It is curious that prior studies have not reported an association between loss of BRCA1 expression and medullary or mucinous histology in nonfamilial breast carcinomas. In addition, the recent immunohistochemical results imply that mechanisms besides promoter hypermethylation may be important in inactivating BRCA1, since 13 (19%) of 69 unselected breast carcinomas and six (17%) of 35 unselected ovarian carcinomas lacked BRCA1 protein expression and many additional cases

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showed greatly reduced expression *(17)*. Esteller et al. *(4)* found that none of the breast cancer cell lines studied displayed BRCA1 promoter hypermethylation, and Wilson et al. *(17)* found that a similar panel of breast cancer cell lines all expressed BRCA1 protein at roughly equivalent levels. While the results support the view that BRCA1 methylation status and expression are related, the observations are puzzling. Perhaps breast cancers lacking BRCA1 expression have proven difficult to establish in *in vitro* culture. Alternatively, spontaneous BRCA1 promoter demethylation and restoration of expression may be selected for during *in vitro* culture. Regardless of the basis for the differences in BRCA1 methylation and expression status between cell lines and primary tumors, the situation renders it essentially impossible to assess the ability of DNA demethylating agents such as 5-azacytidine to rapidly reactivate BRCA1 gene expression in cell lines. This is unfortunate because such data have proven to be important in building the case for the role of methylation as a critical factor in repression of certain tumor suppressor genes in cancer *(1–3)*.

The E-cadherin gene is the topic of the second report by Tamura et al. *(5)* on promoter hypermethylation in cancer. E-Cadherin is a transmembrane glycoprotein that mediates calcium-dependent interactions between adjacent epithelial cells, and loss of E-cadherin expression is a common finding in many human epithelial cancers *(22)*. Recent studies have offered strong evidence that loss of E-cadherin expression plays a causal role in cancer. Germline-inactivating mutations in the E-cadherin gene have been found in families with inherited predisposition to gastric carcinomas, particularly those with diffuse-type histology, and perhaps breast carcinomas *(23–25)*. Somatic mutations in E-cadherin are the most prevalent in lobular breast carcinomas and in diffuse-type gastric carcinomas, with about 50% of the cancers of each of these types displaying somatic mutations inactivating both E-cadherin alleles *(22,26–28)*. However, in most cancers with reduced or absent E-cadherin gene and protein expression, mutations in E-cadherin are rarely detected *(22)*, and proposed mechanisms of E-cadherin inactivation include promoter hypermethylation *(29,30)*, changes in chromatin structure *(31)*, and alterations of specific transcription factor pathways regulating E-cadherin gene expression *(32,33)*.

Tamura et al. *(5)* report that E-cadherin promoter hypermethylation was seen in 27 (51%) of 53 primary gastric carcinomas, including 15 (83%) of 18 undifferentiated (diffuse) type, and E-cadherin promoter hypermethylation was seen at similar frequencies in both early and advanced cases. Similar data on E-cadherin hypermethylation in gastric carcinoma have been offered in a recent study *(34)*, and prior studies *(29,30,35)* have reported on E-cadherin promoter hypermethylation in breast and other cancers. Although it might be attractive to conclude that promoter hypermethylation may be a predominant mechanism of E-cadherin inactivation in gastric and other cancers, unresolved issues cloud the picture. On the basis of prior work demonstrating that somatic mutations and LOH of E-cadherin are present in about half of diffuse-type gastric carcinomas, it would have been particularly valuable for Tamura et al. to have demonstrated that somatic mutations and promoter hypermethylation are mutually exclusive mechanisms of inactivating E-cadherin in gastric carcinoma. The significance of promoter hypermethylation for an E-cadherin allele carrying an inactivating somatic mutation is not clear, and such a finding might question the role of promoter hypermethylation in E-cadherin inactivation. In fact, E-cadherin promoter hypermethylation was not uniformly associated with loss of expression in the study by Tamura et al. *(5)*, since two of the six gastric carcinomas with promoter hypermethylation studied retained E-cadherin expression. Finally, evidence has been presented that E-cadherin expression may be repressed in cancer by mechanisms other than promoter hypermethylation *(32,33)*, and recent work *(36,37)* has shown that the Snail transcription factor may directly repress E-cadherin expression in many epithelial cancers.

Epigenetic mechanisms of gene inactivation, including promoter hypermethylation, are undoubtedly important in cancer development and represent an alternative means of inactivating tumor suppressor genes. Nevertheless, the standard of proof for establishing that hypermethylation of the promoter of any given gene has a critical role in loss of gene expression and cancer development should probably be set quite high, regardless of whether the gene is a well-established tumor suppressor gene, like BRCA1 or E-cadherin, or a potential tumor suppressor gene. Evidence might include data indicating that the methylation status of a promoter is tightly linked to its expression in a large panel of primary cancer specimens and data showing that gene expression can be readily and fully restored by treatment of cancer cells with demethylating agents. In addition, for tumor suppressor genes, evidence that biallelic inactivation of the gene occurs by mutational mechanisms (e.g., localized mutation and LOH) or a combination of mutational and epigenetic mechanisms should be provided. Because several transcription factors that specifically repress tumor suppressor gene expression have been identified, including Snail and its repression of E-cadherin *(36,37)* and the bmi-1 oncoprotein and its repression of $p16^{INK4a}$ *(38)*, it is worth bearing in mind that, in some cases, promoter hypermethylation may be a reflection rather than a cause of gene inactivation in cancer.

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