The Conundrum Posed by Cellular Heterogeneity in Analysis of Human Neuroblastoma

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Examining the methylation patterns of gene promoters has become a powerful tool in the effort to identify and distinguish different tumor subtypes (1,2). Typically associated with tumor suppressor genes, epigenetic silencing of gene expression has been seen in several different cancers. Aberrant hypermethylation has been recorded for genes that function in various aspects of cancer biology, including cell cycle regulation, tumor suppression, apoptosis, and metastasis. The article by Alaminos et al. (3) in this issue is a clear extension of this previous research.

DNA methylation silences gene expression through the addition of methyl groups to cytosine residues of CpG-rich islands present in the promoter region of genes. Whereas housekeeping genes possess CpG regions that are typically unmethylated and therefore transcriptionally active in all cell types, tissue-specific genes are unmethylated in cells actively expressing the genes and are normally methylated in all other differentiated tissues. Patterns of silencing are specified during development; methylation is essential for normal imprinting, X-chromosome inactivation, and differential gene expression during embryonic growth. Changes in DNA methylation patterns have recently been associated with specific cancers, and CpG island hypermethylation profiles for primary tumors have become accepted as a common means to distinguish human cancer subtypes (1,2).

Alaminos et al. (3) initially chose 45 candidate genes representative of important cellular processes and compared their methylation states in 10 human neuroblastoma cell lines and clones by using methylation-specific polymerase chain reaction. The cell lines comprise homogeneous populations of the various cell types—that is, S-type (substrate adherent), N-type (neuroblastic), and I-type (stem) cells—derived from tumors and were used to determine whether specific differences in the methylation profiles of the candidate genes were associated with cell phenotype. I-type cells can differentiate into either neuroblasts or stromal cells. Interestingly, these three distinct cell types differ markedly in malignant potential, with the I-type being the most malignant, as measured by both colony-forming efficiency in soft agar and ability to form tumors in athymic mice (4). Moreover, the relative frequency of putative I-type cells is higher in tumors from patients who relapse than in those who have event-free survival.

Using unbiased cluster analysis to group the datasets, the authors do show that the hypermethylation profiles separate the cell lines into two groups roughly reflecting their MYCN amplification status. This result is potentially important, because MYCN amplification status correlates with malignant potential of tumors. However, amplification is not synonymous with oncogene overexpression, because cell phenotype plays a pivotal role in regulation of both MYCN expression and malignancy (5).

For example, the LA-N-1 cell line, from which both LA1-55n (N-type) and LA1-5s (S-type) were cloned, contains approximately 120 copies of the MYCN gene stably integrated into homogeneously staining regions. Whereas both clonal sublines transcribe the MYCN gene, there is three- to fourfold less mature MYCN mRNA and no oncoprotein in LA1-5s cells compared with LA1-55n cells (5). Moreover, whereas LA1-55n is very malignant, LA1-5s is nontumorigenic. Further complicating the analysis, the SK-N-ER and SH-IN cell lines are I-type clones that are highly tumorigenic, yet they are not MYCN amplified and thus cluster with nontumorigenic SH-EP1 cells (4,5). Consequently, although the results from this small sample of cell lines are informative concerning which genes may be silenced in neuroblastoma, they are insufficient to identify potential targets for therapy or tumorigenic from nontumorigenic cells.

In their second set of experiments, Alaminos et al. used 10 of the candidate genes to generate a DNA hypermethylation profile from 145 human neuroblastoma tumors. The identification of possible tumor suppressor genes in human neuroblastoma has been an important topic of investigation because of the association of a poor prognosis with specific chromosomal deletions; loss of heterozygosity has been demonstrated for chromosomess 1p, 2q, 3p, 9p, 11q, 14q, and 18q (6–10) in subsets of neuroblastomas. These regions, and in particular 1p36, are being studied intensively for possible tumor suppressors. As of yet, no candidate genes have been identified by molecular genetics. More recently, DNA methylation analyses have been used by several groups to identify specific clusters of genes that are hypermethylated and whose pattern of methylation can be used in a prognostic setting. For example, Gonzalez-Gomez et al. (11) studied methylation patterns of 11 genes previously shown to be altered in other cancers in 44 neuroblastoma tumors and attempted to correlate these changes with tumor stage. No specific pattern was evident. Astuti et al. (12) reported promoter hypermethylation of the RASSF1A gene, located on chromosome 3p, in 55% of neuroblastoma tumors, with subsequent loss of expression. However, changes in methylation profile did not correlate with clinical stage (13). Caspase-8 mRNA expression is reduced in neuroblastoma cell lines and linked to promoter hypermethylation (14). van Noesel et al. (15) studied profiles of TRAIL-associated genes in 15 human neuroblastoma cell lines and found that six genes associated with TRAIL sensitivity...
(caspase-8, FLIP, DcR1, DcR2, DR4, and DR5) were hypermethylated in the majority of cell lines. In tumors, only the loss of caspase-8 expression was associated predominantly with high-risk tumors and correlated with MYCN amplification. By contrast, Alaminos et al. identified three genes whose methylation state was associated with prognosis. The success of the present study in distinguishing different risk groups may be a consequence of either the large number of tumors examined or the specific genes chosen for the hypermethylation profile.

A major underlying difficulty contributing to limited detection of specific methylation and gene expression changes in neuroblastoma tumors (and uncultured cell lines) is their cell heterogeneity; the signal-to-noise ratio, i.e., variability in the proportion of N-, S-, and I-type cells within the tumor, may mask real differences in hypermethylation profiles. As noted above, cellular heterogeneity is a hallmark of neuroblastoma tumors and is in fact used to classify tumors into different prognostic stages—tumors that are stroma rich are classified as low-risk tumors, whereas stroma-poor tumors are classified as high risk (16). In addition, the “neuronal” component can differ in extent of maturation, ranging from differentiated or poorly differentiated to undifferentiated cells, with a greater extent of differentiation signifying a better prognosis for the patient. Moreover, preliminary immunocytochemical studies (4) have revealed the presence in all tumors, in various frequencies, of cells analogous to the malignant I-type stem cells of cell lines. Thus, neuroblastoma tumors comprise multiple cell types, with each type theoretically having a distinct hypermethylation and gene expression profile. Even with this inherent variability, methylation profiles may prove prognostic in neuroblastoma and other cancers. However, care is necessary when attributing development of cancer to such changes. Neuroblastoma is a cancer of the developing neural crest in which several of the cell types are pluripotent and can differentiate into the other neuroblastoma cell types. Thus, differences that are seen in hypermethylation profiles in this particular stem cell cancer may reflect changes in methylation accompanying normal genomic restriction rather than development of a cancer phenotype. This consideration may also be important when studying changes in DNA methylation in other cancer types or when analyzing microarray analyses.

Epigenetically mediated regulation of cancer-causing genes by methylation is evident in an increasing number of malignancies (1,2). However, as noted by Baylin and Herman (17), demonstration of promoter hypermethylation by itself does not demonstrate tumor suppressor gene function. Clearly, we are just starting down the road to understanding the molecular biology of tumors and to identifying the genes that may be involved in the many divergent pathways that lead to malignancy and metastasis.

**References**