Abstract

We are in an era where the potential exists for deriving comprehensive profiles of DNA alterations characterizing each form of human cancer. Such profiles would provide invaluable insight into mechanisms underlying the evolution of each tumor type and will provide molecular markers, which could radically improve cancer detection. To date, no one type of DNA change has been defined which accomplishes this purpose. Herein, by using a candidate gene approach, we show that one category of DNA alteration, aberrant methylation of gene promoter regions, can enormously contribute to the above goals. We have now analyzed a series of promoter hypermethylation changes in 12 genes (p16\textsuperscript{INK4a}, p15\textsuperscript{INK4b}, p14\textsuperscript{ARF}, p73, APC,\textsuperscript{2} BRCA1, hMLH1, GSTP1, MGMT, CDH1, TIMP3, and DAPK), each rigorously characterized for association with abnormal gene silencing in cancer, in DNA from over 600 primary tumor samples representing 15 major tumor types. The genes play known important roles in processes encompassing tumor suppression, cell cycle regulation, apoptosis, DNA repair, and metastatic potential. A unique profile of promoter hypermethylation exists for each human cancer in which some gene changes are shared and others are cancer-type specific. The hypermethylation of the genes occurs independently to the extent that a panel of three to four markers defines an abnormality in 70–90% of each cancer type. Our results provide an unusual view of the pervasiveness of DNA alterations, in this case an epigenetic change, in human cancer and a powerful set of markers to outline the disruption of critical pathways in tumorigenesis and for derivation of sensitive molecular detection strategies for virtually every human tumor type.

Unfolding a Gene Hypermethylation Profile of Human Cancer

Mutations in individual genes have outlined critical aspects of tumorigenesis, including disruption of the Rb, p16\textsuperscript{INK4a}, APC\textsuperscript{3} β-catenin/\textit{Tcf}, and p53/p14\textsuperscript{ARF}/MDM2 pathways. Global genome screens, such as for repeat microsatellite sequence alterations and for gene expression changes by serial analysis of gene expression (1) or cDNA microarrays (2), have also provided important information about molecular events important for tumorigenesis. Despite these above studies, no detection of any one type of DNA alteration, either by candidate gene approach or by genomic screening techniques, has provided universal markers for all tumor types. In the present study, we demonstrate how one single type of DNA alteration, aberrant methylation of gene promoters, can point to pathways disrupted in every type of cancer and can provide markers for sensitive detection of virtually all tumor types.

The growing list of genes inactivated by promoter region hypermethylation provides an opportunity to examine the patterns of inactivation of such genes among different tumors (3, 4). Recently, a global pattern of methylation events in tumors using restriction landmark genomic scanning was reported (5). We have instead used a candidate gene approach. We have studied multiple key cancer genes undergoing epigenetic inactivation in a large set of primary human tumors with the aim of obtaining a map of this alteration in malignant transformation. A total of 12 genes, including well-characterized tumor suppressor genes (p16\textsuperscript{INK4a}, p15\textsuperscript{INK4b}, p14\textsuperscript{ARF}, p73, APC, and BRCA1), DNA repair genes (hMLH1, GSTP1, and MGMT), and genes related to metastasis and invasion (CDH1, TIMP3, and DAPK) were included in the study. Each of these genes possesses a CpG island in their 5’ region which is unmethylated in corresponding normal tissues, as expected for a typical CpG island (6). We and others have shown, in previous studies for such genes in individual tumor types, that when these CpG islands are hypermethylated in cancer cells, expression of the corresponding gene is silenced and the silencing can be partially relieved by demethylation of the promoter region (3, 4). However, the scope of these changes has not been easy to observe by examining each study individually.

The primary tumor samples examined in the present study constitute over 600 specimens that cover 15 major tumor types (colon, stomach, pancreas, liver, kidney, lung, head and neck, breast, ovary, endometrium, kidney, bladder, brain, and lymphomas). The profile of promoter hypermethylation for each of the above genes in each tumor type is shown in Fig. 1. Important features of the data are as follows. First, one or more of the genes studied is hypermethylated in every tumor type. However, the profile of promoter hypermethylation for the genes differs for each cancer type, providing a tumor-type and gene-specific profile. Some genes, such as the cell cycle inhibitor p16\textsuperscript{INK4a}, are hypermethylated across many tumor types including colorectal, lung, and breast carcinomas as previously described (7–9). This alteration reflects the widespread contribution of disruptions of the cyclinD-Rb cell cycle control pathway in human cancer. The extent of p16\textsuperscript{INK4a} epigenetic silencing reported in the literature expands over the neoplasms described in Fig. 1 and also include bladder (10) and cervical tumors (11) or melanomas (12) and gliomas (13).

Other changes, such as for the DNA repair gene MGMT and DAPK, also have a wide distribution (14–17). Hypermethylation of p14\textsuperscript{ARF} and APC are most prevalent in gastrointestinal tumors (i.e., colon and stomach) (18, 19), whereas GSTP1 is characteristic of steroid-related neoplasms such as breast, liver, and prostate (20, 21). The mentioned spectrum of epigenetic inactivation we have observed has been confirmed in other laboratories studying a single tumor type such as MGMT (22) and APC (23) in colorectal, p14\textsuperscript{ARF} in gastric tumors (24), DAPK in bladder tumors (25), and GSTP1 in hepatic neoplasms (26).

The aberrant methylation of certain genes reflects their very specific involvement in selected tumor types or groups of tumors. Thus, as might be predicted from the tumor types seen in families who...
inherit mutations in BRCA1, we found hypermethylation of this gene only in breast and ovarian carcinomas (27), consistent with other reports (28), whereas hypermethylation of the mismatch repair gene hMLH1 is restricted to the three sporadic tumor types characteristic of the hereditary nonpolyposis colorectal cancer syndrome: colorectal, endometrial, and gastric tumors enriched in microsatellite-unstable samples (29–32). Similarly, hypermethylation of p73 and p15 INK4b is only observed in hematological malignancies (33–37).

Another interesting point is raised by the fact that epigenetic inactivation may affect all of the molecular pathways involved in cell immortalization and transformation. We observed promoter hypermethylation-associated silencing in cell cycle (p16 INK4a and p15 INK4b), DNA repair (hMLH1, MGMT, and BRCA1), cell adherence and metastasis process (CDH1, TIMP3, DAPK), p53 network (p14 ARF and p73), metabolic enzymes (GSTP1), and the APC/b-catenin route (APC). Thus, in any given tumor it is possible to find simultaneous inactivation of several pathways by aberrant methylation compromising all of the described function; i.e., a colorectal tumor may have disruption of cell cycle, DNA repair, and metastasis-related process by hypermethylation of p16 INK4a, hMLH1, and CDH1, respectively, whereas a mammary tumor can accomplish similar objectives silencing p16 INK4a, BRCA1, and CDH1 and a lung tumor affecting p16 INK4a, MGMT, and DAPK.

If we look at our gene hypermethylation profile from the tumor type standpoint, the scenario is particularly interesting. Gastrointestinal tumors (colon and gastric) share a set of genes undergoing hypermethylation characterized by p16INK4a, p14ARF, MGMT, APC, and hMLH1, whereas other aerodigestive tumor types, such as lung and head and neck, have a different pattern of hypermethylated genes including DAPK, MGMT, and p16INK4a, but not hMLH1 or p14ARF. Similarly, breast and ovarian cancers tend to methylate certain genes including BRCA1, GSTP1, and p16 INK4a, but not hMLH1 or p14 ARF.

In each case and tumor type, these epigenetic lesions occur in the absence of a genetic lesion. A couple of illustrative examples are found in colorectal tumorigenesis. First, while homozygous deletion of the INK4a/ARF are common in other tumor types, this genetic abrogation is uncommon in colon tumors and instead this locus is commonly shutdown by simultaneous methylation of p16INK4a and p14ARF (8, 9, 18, 43). Second, because APC somatic mutation is very prevalent in these tumors, APC methylation is observed at a low frequency, but other gastrointestinal tumors types, that usually do not harbor APC mutations, can disrupt the APC/b-catenin pathway through APC hypermethylation (19, 39).

Furthermore, the presence of the epigenetic lesion is often an early
event in the natural history of human cancer. Promoter hypermethylation affecting \( p16^{INK4A} \), \( p14^{ARF} \), \( MGMT \), and \( APC \) occurs in colorectal adenomas (15, 18, 19), \( p16^{INK4A} \) hypermethylations is detectable in basal cell hyperplasia squamous metaplasia and carcinoma in situ of the lung (44), and \( hMLH1 \) epigenetic silencing can be demonstrated in endometrial hyperplasias (45) and ulcerative colitis (46), both precursor lesions of uterine and colorectal tumors.

This analysis of candidate genes can be seen as only a partial picture of the methylation changes in cancer. First, there are certainly still numerous genes that undergo epigenetic inactivation waiting to be discovered. The completion of the human genome sequence and the use of several described techniques to find new genes with differential methylation, such as methylation-sensitive arbitrarily primed PCR (47), methylated CpG island amplification (48), restriction landmark genomic scanning (5, 49), and differential methylation hybridization (50) will be extremely useful for this purpose. Examples of genes found by these and other approaches include genes such as \( TPEF \) (51) or the proapoptotic \( TMS1 \) (52), and future studies will likely address their distribution and relevance in multiple tumor types.

**Insights into a Molecular Marker System for Cancer Based on Aberrant Methylation**

A major possibility raised by our current data are that promoter hypermethylation changes might provide a molecular marker system for the detection of the major forms of human cancer. This DNA change is obviously common to each tumor type studied and the frequency for hypermethylation of many of the genes, determined by tumor type, is often high. In fact, for each tumor type studied, three or more of the genes tested were hypermethylated in at least 5–10% of the samples tested and often many more. For ease of detection, the promoter hypermethylation may offer many advantages as compared to other DNA alterations such as mutations. These latter changes often occur at different sites, even for point mutations within a given gene, between individual tumors even of the same type. Promoter hypermethylation, in contrast, occurs over the same regions of a given gene in each form of cancer. Thus, one need not first test the methylation status of a given gene in tumor DNA to devise means for detecting the hypermethylation marker in DNA from a distal site. Finally, as compared to other frequent chromosome changes in cancer, such as allelic losses, the hypermethylation constitutes a positive signal, which is easier to detect against a background of normal DNA. With regard to all of the above points, a number of studies, using sensitive PCR strategies for detection of promoter hypermethylation changes in specific genes, provide proof of principle that these changes can be used to detect cancer through analyses of DNA from readily obtainable sites such as serum and sputum (16, 17, 53).

To test the diagnostic potential of our findings for the 12 genes under study, we picked a subset of genes, selected according to the frequency data in Fig. 1, of hypermethylated genes for each of five tumor types. To test the feasibility of this approach, we first explored whether hypermethylation for each constitutive gene in the panel is an independent event (Fig. 2). This would be necessary to obtain maximum coverage of each cancer type using a minimum number of markers to assay. Indeed, for each cancer type (Fig. 2), the incidence of hypermethylation for zero, one, two, three, and four genes was not statistically different from these changes being randomly associated (\( P = 0.38–0.97 \)). Most important, we detected changes in at least one of these genes in approximately 80% or more of the samples from each tumor type (Fig. 2).

The use of methylation markers for the detection of transformed cells is not only a black and white tool, but also a qualitative one. According to the profile of genes whose hypermethylation-associated inactivation is detected, we may have important information about the biological behavior of that particular malignancy. Two sets of genes are noteworthy to mention: the DNA repair and the cell adherence group. First, we have previously demonstrated that transcriptional inactivation of \( MGMT \) by methylation occurs in a wide spectrum of human tumors (14). We have now shown that \( MGMT \) epigenetic
siliencing in gliomas confers enhanced sensitivity to alkylating drugs (54). Similar results have also been suggested in the past to be related to the action of hMLH1 and GSTP1 in relation to other agents (55, 56). Thus, the combined methylation analysis of these three genes may contribute to predict which chemotherapy would be more effective in each cancer patient. Second, the test of the epigenetic status of CDH1, TIMP3, and DAPK may provide us with a valuable measure of the metastatic potential of any given tumor (57). Because aberrant methylation can occur when the cancer cells have not yet spread, this knowledge could be used to try treatments to prevent dissemination in these aggressive tumors.

Conclusions

Overall, our data demonstrate, using a candidate gene approach, that promoter hypermethylation of 12 genes involving important cellular pathways in tumorigenesis is a feature of each of 15 major human tumor types studied. Moreover, although many tumors share this change for a given gene, unique profiles do exist for the tumor types. Finally, small panels of hypermethylated gene markers can detect a high percentage of each of the tumor types studied. Thus, the spectrum of epigenetic alterations for a relatively small subset of genes provides a potentially powerful system of biomarkers for developing molecular detection strategies for virtually every form of human cancer.

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