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Perspectives in Cancer Research

A Gene Hypermethylation Profile of Human Cancer[1]

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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 (*$p16^{INK4a}$*, *$p15^{INK4b}$*, *$p14^{ARF}$*, *p73*, *APC*,[5] *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 metastastic 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*/*$p16^{INK4a}$*, *APC*/[5]*β-catenin*/*Tcf*, and *p53*/*$p14^{ARF}$*/*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 (*$p16^{INK4a}$*, *$p15^{INK4b}$*, *$p14^{ARF}$*, *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 leukemia 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 *$p16^{INK4a}$*, 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 $p16^{INK4a}$ 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 *$p14^{ARF}$* 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, *$p14^{ARF}$* 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

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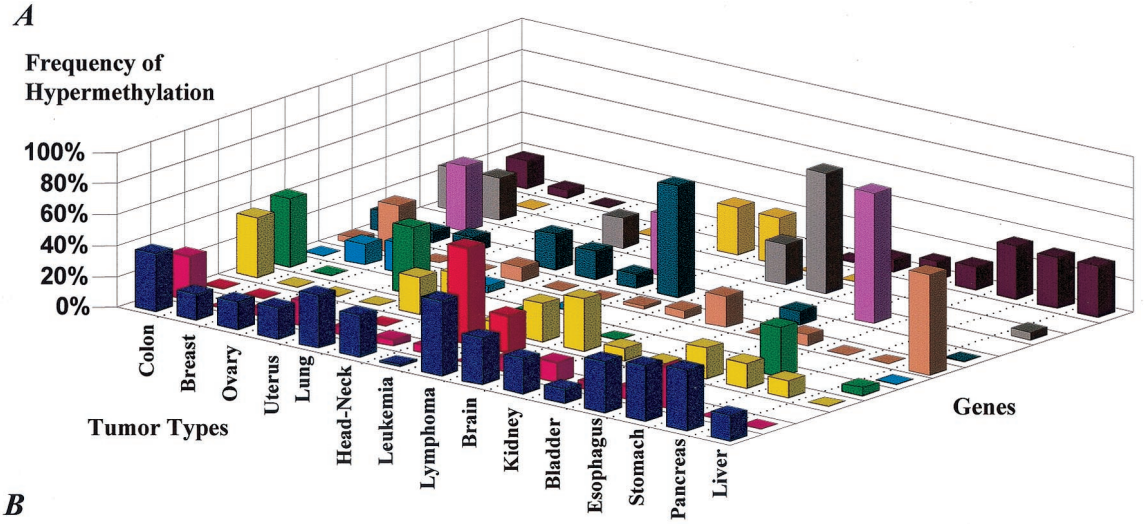
[5] The abbreviations used are: APC, adenomatous polyposis coli; CDH1, E-cadherin; DAPK, death-associated protein kinase; GSTP1, glutathione *S*-transferase P1; MGMT, O^6-methylguanine-DNA methyltransferase; TIMP3, tissue inhibitor of metalloproteinase-3.

A



B

	p16 INK4a	p14 ARF	p15 INK4b	MGMT	hMLH1	BRCA1	GSTP1	DAPK	CDH1	TIMP-3	p73	APC
Colon	37%,41/110	28%,37/132	0%,0/19	39%,127/323	44%,15/34*	0%,0/18	4%,1/23	13%,2/23	N.D.	27%,6/22	0%,0/10	18%,20/108
Breast	17%,11/66	0%,0/20	0%,0/16	0%,0/36	0%,0/10	13%,11/84	31%,24/77	7%,1/15	42%,37/88	27%,8/29	0%.0/15	5%,1/19
Ovary	18%,4/22	5%,1/20	N.D.	0%,0/23	N.D.	19%,11/58	0%,0/10	9%,2/23	N.D.	N.D.	N.D.	0%,0/20
Uterus	20%,6/29	16%,4/25	N.D.	0%,0/17	43%,24/56*	N.D.	0%,0/20	N.D.	N.D.	N.D.	N.D.	N.D.
Lung	31%,28/89	6%,4/62	0%,0/21	21%,18/83	0%,0/20	4%,1/22	9%,2/21	16%,10/64	N.D.	19%,4/21	0%,0/22	0%,0/17
Head-Neck	27%,26/95	4%,1/25	N.D.	32%,37/116	N.D.	N.D.	0%,0/106	18%,17/92	N.D.	N.D.	N.D.	0%,0/10
Leukemia	1%,1/150	5%,1/20	62%,93/150	6%,2/31	6%,3/51	0%,0/19	0%,0/10	9%,8/86	40%,30/75	N.D.	31%,11/35	N.D.
Lymphoma	48%,12/25	0%,0/22	24%,6/25	25%,15/61	N.D.	N.D.	2%,1/47	72%,21/29	N.D.	N.D.	30%,3/10	N.D.
Brain	30%,3/10	9%,2/22	N.D.	34%,74/213	0%,0/15	N.D.	5%,1/20	N.D.	N.D.	26%,20/77	0%,0/22	0/10
Kidney	23%,6/25	13%,5/38	N.D.	8%,1/12	N.D.	N.D.	20%,8/35	N.D.	N.D.	78%,28/36	0%,0/10	8%,1/12
Bladder	9%,1/11	5%,1/20	N.D.	4%,2/44	N.D.	N.D.	0%,0/24	9%,1/11	N.D.	N.D.	N.D.	10%,2/19
Esophagus	33%,5/15	8%,3/37	N.D.	20%,3/14	N.D.	N.D.	7%,1/14	N.D.	84%,26/31	N.D.	N.D.	15%,4/27
Stomach	36%,8/22	26%,31/118	N.D.	16%,10/60	32%,21/65*	N.D.	0%,0/22	N.D.	N.D.	N.D.	N.D.	34%,13/38
Pancreas	39%,7/18	0%,0/20	N.D.	11%,2/18	N.D.	N.D.	0%,0/18	N.D.	N.D.	N.D.	N.D.	33%,6/18
Liver	15%,3/20	0%,0/20	N.D.	0%,0/59	5%,2/20	0%,0/18	65%,13/20	0%,0/20	N.D.	5%,1/20	N.D.	33%,6/18

Fig. 1. *A*, depiction of the profile of gene promoter hypermethylation across human tumor types. All cases represent random and unselected populations of each particular tumor type, except ∗, where hMLH1 methylation was determined in colorectal, endometrial, and gastric tumors enriched in microsatellite-unstable samples. Analysis of the methylation status was studied in most cases by sodium bisulfite modification of DNA and subsequent PCR using primers designed for either methylated or unmethylated DNA (PCR conditions and sequences are available upon request). Additional samples were analyzed by Southern blot with methyl-sensitive enzymes, restriction cut analysis, and bisulfite genomic sequencing. *B*, numerical distribution of promoter hypermethylation according to gene and tumor type.

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 with microsatellite instability (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/β-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 *TIMP-3*, 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 $p16^{INK4a}$, $p14^{ARF}$, *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 $p16^{INK4a}$, but not *hMLH1* or $p14^{ARF}$. Similarly, breast and ovarian cancers tend to methylate certain genes including *BRCA1*, *GSTP1*, and $p16^{INK4a}$. This gene hypermethylation profile of human cancer that we report is consistent with the data of particular "methylotypes" proposed for single tumor types including tumors originated from the pancreas, esophagus, stomach, colon, and leukemia (38–42). It is noteworthy that hematological malignancies have markedly different epigenetic alterations than do tumors originating in solid organs. This is evident in the high frequency of *p73* and $p15^{INK4b}$ hypermethylation in these tumors while these genes are not altered in the epithelial tumors.

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 $p16^{INK4a}$ and $p14^{ARF}$ (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/β-catenin* pathway through *APC* hypermethylation (19, 39).

Furthermore, the presence of the epigenetic lesion is often an early

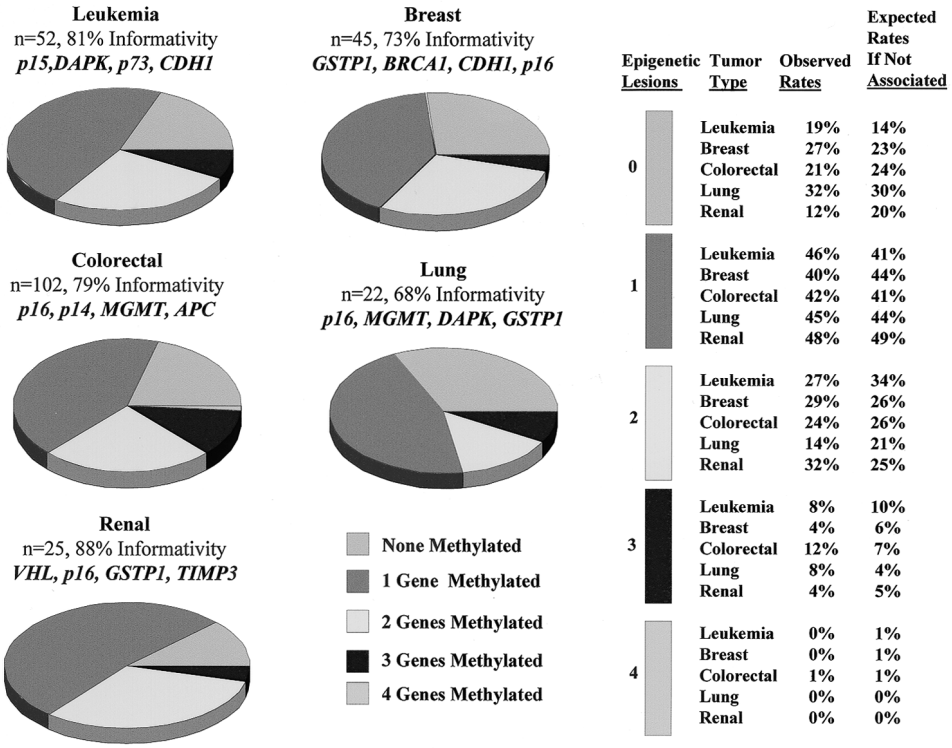


Fig. 2. *Left*, depiction of coincident number of gene hypermethylation events in a defined tumor. *Right*, predicted detection rate if the changes are independent events *versus* linked events and how this impacts on the percentage of a given tumor type that a panel of markers would pick up.

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 events ($P = 0.38-0.97$). 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

silencing 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 metastastic 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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References

1. Velculescu, V. E., Zhang, L., Vogelstein, B., and Kinzler, K. W. Serial analysis of gene expression. Science (Wash DC), *270:* 484–487, 1995.
2. Ross, D. T., Scherf, U., Eisen, M. B., Perou, C. M., Rees, C., Spellman, P., Iyer, V., Jeffrey, S. S., Van de Rijn, M., Waltham, M., Pergamenschikov, A., Lee, J. C., Lashkari, D., Shalon, D., Myers, T. G., Weinstein, J. N., Botstein, D., and Brown, P. O. Systematic variation in gene expression patterns in human cancer cell lines. Nat. Genet., *24:* 227–235, 2000.
3. Jones, P. A., and Laird, P. W. Cancer epigenetics comes of age. Nat. Genet., *21:* 163–167, 1999.
4. Baylin, S. B., and Herman, J. G. DNA hypermethylation in tumorigenesis: epigenetics joins genetics. Trends Genet., *16:* 168–174, 2000.
5. Costello, J. E. Fruhwald, M. C., Smiraglia, D. J., Rush, L. J., Robertson, G. P., Gao, X., Wright, F. A., Feramisco, J. D., Peltomaki, P., Lang, J. C., Schuller, D. E., Yu, L., Bloomfield, C. D., Caligiuri, M. A., Yates, A., Nishikawa, R., Su Huang, H., Petrelli, N. J., Zhang, X., O'Dorisio, M. S., Held, W. A., Cavenee, W. K., and Plass, C. Aberrant CpG-island methylation has nonrandom and tumour-type-specific patterns. Nat. Genet., *24:* 132–138, 2000.
6. Bird, A. P., and Wolffe, A. P. Methylation-induced repression–belts, braces, and chromatin. Cell, *99:* 451–454, 1999.
7. Merlo, A, Herman, J. G., Mao, L., Lee, D. J., Gabrielson, E., Burger, P. C., Baylin, S. B., and Sidransky, D. 5′ CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. Nat. Med., *1:* 686–692, 1995.
8. Herman, J. G., Merlo, A., Mao, L., Lapidus, R. G., Issa, J. P., Davidson, N. E., Sidransky, D., and Baylin, S. B. Inactivation of the *CDKN2/p16/MTS1* gene is frequently associated with aberrant DNA methylation in all common human cancers. Cancer Res., *55:* 4525–4530, 1995.
9. Gonzalez-Zulueta, M., Bender, C. M., Yang, A. S., Nguyen, T., Beart, R. W., Van Tornout, J. M., and Jones, P. A. Methylation of the 5′ CpG island of the *p16/CDKN2* tumor-suppressor gene in normal and transformed human tissues correlates with gene silencing. Cancer Res., *55:* 4531–4535, 1995.
10. Salem, C., Liang, G., Tsai, Y. C., Coulter, J., Knowles, M. A., Feng, A. C., Groshen, S., Nichols, P. W., and Jones, P. A. Progressive increases in de novo methylation of CpG islands in bladder cancer. Cancer Res., *60:* 2473–2476, 2000.
11. Nuovo, G. J., Plaia, T. W., Belinsky, S. A., Baylin, S. B., and Herman, J. G. In situ detection of the hypermethylation-induced inactivation of the *p16* gene as an early event in oncogenesis. Proc. Natl. Acad. Sci. USA, *96:* 12754–12759, 1999.
12. Gonzalgo, M. L., Bender, C. M., You, E. H., Glendening, J. M., Flores, J. F., Walker, G. J., Hayward, N. K., Jones, P. A., and Fountain, J. W. Low frequency of p16/CDKN2A methylation in sporadic melanoma: comparative approaches for methylation analysis of primary tumors. Cancer Res., *57:* 5336–5347, 1997.
13. Costello, J. F., Berger, M. S., Huang, H. S., and Cavenee, W. K. Silencing of p16/CDKN2 expression in human gliomas by methylation and chromatin condensation. Cancer Res., *56:* 2405–2410, 1996.
14. Esteller, M., Hamilton, S. R., Burger, P. C., Baylin, S. B., and Herman, J. G. Inactivation of the DNA repair gene O^6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. Cancer Res., *59:* 793–797, 1999.
15. Esteller, M., Toyota, M., Sanchez-Cespedes, M., Capella, G., Peinado, M. A., Watkins, D. N., Issa, J. P., Sidransky, D., Baylin, S. B., and Herman, J. G. Inactivation of the DNA repair gene O^6-methylguanine-DNA methyltransferase by promoter hypermethylation is associated with G to A mutations in K-*ras* in colorectal tumorigenesis. Cancer Res., *60:* 2368–2371, 2000.
16. Esteller, M., Sanchez-Cespedes, M., Rosell, R., Sidransky, D., Baylin, S. B., and Herman, J. G. Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from nonsmall cell cancer patients. Cancer Res., *59:* 67–70, 1999.
17. Sanchez-Cespedes, M., Esteller, M., Wu, L., Nawroz-Danish, H., Yoo, G. H., Koch, W. M., Jen, J., Herman, J. G., and Sidransky, D. Gene promoter hypermethylation in tumors and serum of head and neck cancer patients. Cancer Res., *60:* 892–895, 2000.
18. Esteller, M., Tortola, S., Toyota, M., Capella, G., Peinado, M. A., Baylin, S. B., and Herman, J. G. Hypermethylation-associated inactivation of $p14^{ARF}$ is independent of $p16^{INK4a}$ methylation and *p53* mutational status. Cancer Res., *60:* 129–133, 2000.
19. Esteller, M., Sparks, A., Toyota, M., Sanchez-Cespedes, M., Capella, G., Peinado, M. A., Gonzalez, S., Tarafa, G., Sidransky, D., Meltzer, S. J., Baylin, S. B., and Herman, J. G. Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer. Cancer Res., *60:* 4366–4371, 2000.
20. Esteller, M., Corn, P. G., Urena, J. M., Gabrielson, E., Baylin, S. B., and Herman, J. G. Inactivation of glutathione *S*-transferase *P1* gene by promoter hypermethylation in human neoplasia. Cancer Res., *5:* 4515–4518, 1998.
21. Lee, W. H., Morton, R. A., Epstein, J. I., Brooks, J. D., Campbell, P. A., Bova, G. S., Hsieh, W. S., Isaacs, W. B., and Nelson, W. G. Cytidine methylation of regulatory sequences near the pi-class glutathione *S*-transferase gene accompanies human prostatic carcinogenesis. Proc. Natl. Acad. Sci. USA, *91:* 11733–11737, 1994.
22. Herfarth, K. K., Brent, T. P., Danam, R. P., Remack, J. S., Kodner, I. J., Wells, S. A., Jr., and Goodfellow, P. J. A specific CpG methylation pattern of the MGMT promoter region associated with reduced MGMT expression in primary colorectal cancers. Mol. Carcinog., *24:* 90–98, 1999.
23. Hiltunen, M. O., Alhonen, L., Koistinaho, J., Myohanen, S., Paakkonen, M., Marin, S., Kosma, V. M., and Janne, J. Hypermethylation of the *APC* (*adenomatous polyposis coli*) gene promoter region in human colorectal carcinoma. Int. J. Cancer, *70:* 644–648, 1997.
24. Iida, S., Akiyama, Y., Nakajima, T., Ichikawa, W., Nihei, Z., Sugihara, K., and Yuasa, Y. Alterations and hypermethylation of the $p14^{ARF}$ gene in gastric cancer. Int. J. Cancer, *87:* 654–658, 2000.
25. Kissil, J. L., Feinstein, E., Cohen, O., Jones, P. A., Tsai, Y. C., Knowles, M. A., Eydmann, M. E,., and Kimchi, A. DAP-kinase loss of expression in various carcinoma and B-cell lymphoma cell lines: possible implications for role as tumor suppressor gene. Oncogene, *15:* 403–407, 1997.
26. Tchou, J. C., Lin, X., Freije, D., Isaacs, W. B., Brooks, J. D., Rashid, A., De Marzo, A. M., Kanai, Y., Hirohashi, S., and Nelson, W. G. GSTP1 CpG island DNA hypermethylation in hepatocellular carcinomas. Int. J. Oncol. *16:* 663–676, 2000.
27. Esteller, M., Silva, J. M., Dominguez, G., Bonilla, F., Matias-Guiu, X., Lerma, E., Bussaglia, E., Prat, J., Harkes, I. C., Repasky, E. A., Gabrielson, E., Schutte, M., Baylin, S. B., and Herman, J. G. Promoter hypermethylation and *BRCA1* inactivation in sporadic breast and ovarian tumors. J. Natl. Cancer Inst., *92:* 564–569, 2000.
28. Bianco, T., Chenevix-Trench, G., Walsh, D. C., Cooper, J. E., and Dobrovic, A. Tumour-specific distribution of *BRCA1* promoter region methylation supports a pathogenetic role in breast and ovarian cancer. Carcinogenesis (Lond.), *21:* 147–151, 2000.
29. Kane, M. F., Loda, M., Gaida, G. M., Lipman, J., Mishra, R., Goldman, H., Jessup, J. M., and Kolodner, R. Methylation of the *hMLH1* promoter correlates with lack of expression of *hMLH1* in sporadic colon tumors and mismatch repair-defective human tumor cell lines. Cancer Res., *57:* 808–811, 1997.
30. Herman, J. G., Umar, A., Polyak, K., Graff, J. R., Ahuja, N., Issa, J. P., Markowitz, S., Willson, J. K., Hamilton, S. R., Kinzler, K. W., Kane, M. F., Kolodner, R. D., Vogelstein, B., Kunkel, T. A., and Baylin, S. B. Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. Proc. Natl. Acad. Sci. USA, *95:* 6870–6875, 1998.
31. Esteller, M., Levine, R., Baylin, S. B., Ellenson, L. H., and Herman, J. G. *MLH1* promoter hypermethylation is associated with the microsatellite instability phenotype in sporadic endometrial carcinomas. Oncogene, *17:* 2413–2417, 1998.
32. Fleisher, A. S., Esteller, M., Wang, S., Tamura, G., Suzuki, H., Yin, J., Zou, T. T., Abraham, J. M., Kong, D., Smolinski, K. N., Shi, Y. Q., Rhyu, M. G., Powell, S. M., James, S. P., Wilson, K. T., Herman, J. G., and Meltzer, S. J. Hypermethylation of the *hMLH1* gene promoter in human gastric cancers with microsatellite instability. Cancer Res., *59:* 1090–1095, 2000.
33. Corn, P. G., Kuerbitz, S. J., van Noesel, M. M., Esteller, M., Compitello, N., Baylin, S. B., and Herman, J. G. Transcriptional silencing of the *p73* gene in acute lymphoblastic leukemia and Burkitt's lymphoma is associated with 5′ CpG island methylation. Cancer Res., *59:* 3352–3356, 1999.

34. Kawano, S., Miller, C. W., Gombart, A. F., Bartram, C. R., Matsuo, Y., Asou, H., Sakashita, A., Said, J., Tatsumi, E., and Koeffler, H. P. Loss of *p73* gene expression in leukemias/lymphomas due to hypermethylation. Blood, *94:* 1113–1120, 1999.
35. Herman, J. G., Jen, J., Merlo, A., and Baylin, S. B. Hypermethylation-associated inactivation indicates a tumor suppressor role for $p15^{INK4B}$. Cancer Res., *56:* 722–727, 1996.
36. Batova, A., Diccianni, M. B., Yu, J. C., Nobori, T., Link, M. P., Pullen, J., and Yu, A. L. Frequent and selective methylation of p15 and deletion of both p15 and p16 in T-cell acute lymphoblastic leukemia. Cancer Res., *57:* 832–836, 1997.
37. Wong, I. H., Ng, M. H., Huang, D. P., and Lee, J. C. Aberrant p15 promoter methylation in adult and childhood acute leukemias of nearly all morphologic subtypes: potential prognostic implications. Blood, *95:* 1942–1949, 2000.
38. Ueki, T., Toyota, M., Sohn, T., Yeo, C. J., Issa, J. P., Hruban, R. H., and Goggins, M. Hypermethylation of multiple genes in pancreatic adenocarcinoma. Cancer Res., *60:* 1835–1839, 2000.
39. Eads, C. A., Lord, R. V., Kurumboor, S. K., Wickramasinghe, K., Skinner, M. L., Long, T. I., Peters, J. H., DeMeester, T. R., Danenberg, K. D., Danenberg, P. V., Laird, P. W., and Skinner, K. A. Fields of aberrant CpG island hypermethylation in Barrett's esophagus and associated adenocarcinoma. Cancer Res., *60:* 5021–5026, 2000.
40. Toyota, M., Ahuja, N., Suzuki, H., Itoh, F., Ohe-Toyota, M., Imai, K., Baylin, S. B., and Issa, J. P. Aberrant methylation in gastric cancer associated with the CpG island methylator phenotype. Cancer Res., *59:* 5438–5442, 1999.
41. Toyota, M., Ahuja, N., Ohe-Toyota, M., Herman, J. G., Baylin, S. B., and Issa, J. P. CpG island methylator phenotype in colorectal cancer. Proc. Natl. Acad. Sci. USA, *96:* 8681–8686, 1999.
42. Melki, J. R., Vincent, P. C., and Clark, S. J. Concurrent DNA hypermethylation of multiple genes in acute myeloid leukemia. Cancer Res., *59:* 3730–3740, 1999.
43. Robertson, K. D., and Jones, P. A. The human ARF cell cycle regulatory gene promoter is a CpG island which can be silenced by DNA methylation and down-regulated by wild-type p53. Mol. Cell. Biol., *18:* 6457–6473, 1998.
44. Belinsky, S. A., Nikula, K. J., Palmisano, W. A., Michels, R., Saccomanno, G., Gabrielson, E., Baylin, S. B., and Herman, J. G. Aberrant methylation of $p16^{INK4a}$ is an early event in lung cancer and a potential biomarker for early diagnosis. Proc. Natl. Acad. Sci. USA, *95:* 11891–11896, 1998.
45. Esteller, M., Catasus, L., Matias-Guiu, X., Mutter, G. L., Prat, J., Baylin, S. B., and Herman, J. G. hMLH1 promoter hypermethylation is an early event in human endometrial tumorigenesis. Am. J. Pathol., *155:* 1767–1772, 1999.
46. Fleisher, A. S., Esteller, M., Harpaz, N., Leytin, A., Rashid, A., Xu, Y., Liang, J., Stine, O. C., Yin, J., Zou, T. T., Abraham, J. M., Kong, D., Wilson, K. T., James, S. P., Herman, J. G., and Meltzer, S, J. Microsatellite instability in inflammatory bowel disease-associated neoplastic lesions is associated with hypermethylation and diminished expression of the DNA mismatch repair gene, *hMLH1*. Cancer Res., *60:* 4864–4868, 2000.
47. Gonzalgo, M. L., Liang, G., Spruck, C. H., III, Zingg, J. M., Rideout, W. M., III, and Jones, P. A. Identification and characterization of differentially methylated regions of genomic DNA by methylation-sensitive arbitrarily primed PCR. Cancer Res., *57:* 594–599, 1997.
48. Toyota, M., Ho, C., Ahuja, N., Jair, K. W., Li, Q., Ohe-Toyota, M., Baylin, S. B., and Issa, J. P. Identification of differentially methylated sequences in colorectal cancer by methylated CpG island amplification. Cancer Res., *59:* 2307–2312, 1999.
49. Hayashizaki, Y., Hirotsune, S., Okazaki, Y., Hatada, I., Shibata, H., Kawai, J., Hirose, K., Watanabe, S., Fushiki, S., and Wada, S. Restriction landmark genomic scanning method and its various applications. Electrophoresis, *14:* 251–258, 1993.
50. Huang, T. H., Perry, M. R., and Laux, D. E. Methylation profiling of CpG islands in human breast cancer cells. Hum. Mol. Genet., *8:* 459–470, 1999.
51. Liang, G., Robertson, K. D., Talmadge, C., Sumegi, J., and Jones, P. A. The gene for a novel transmembrane protein containing epidermal growth factor and follistatin domains is frequently hypermethylated in human tumor cells. Cancer Res., *60:* 4907–4912, 2000.
52. Conway, K. E., McConnell, B. B., Bowring, C. E., Donald, C. D., Warren, S. T., and Vertino, P. M. TMS1, a novel proapoptotic caspase recruitment domain protein, is a target of methylation-induced gene silencing in human breast cancers. Cancer Res., *60:* 6236–6242, 2000.
53. Palmisano, W. A., Divine, K. K., Saccomanno, G., Gilliland, F. D., Baylin, S. B., Herman, J. G., and Belinsky, S. A. Predicting lung cancer by detecting aberrant promoter methylation in sputum. Cancer Res., *60:* 5954–5958, 2000.
54. Esteller, M., Garcia-Foncillas, J., Andion, E., Goodman, S. N., Hidalgo, O. F., Vanaclocha, V., Baylin, S. B., and Herman, J. G. Inactivation of the DNA repair gene *MGMT* and the clinical response of gliomas to alkylating agents. N. Engl. J. Med., *343:* 1350–1354, 2000.
55. Strathdee, G., MacKean, M. J., Illand, M., and Brown, R. A role for methylation of the hMLH1 promoter in loss of hMLH1 expression and drug resistance in ovarian cancer. Oncogene, *18:* 2335–2341, 1999.
56. Tew, K. D. Glutathione-associated enzymes in anticancer drug resistance. Cancer Res., *54:* 4313–4320, 1994.
57. Tang, X., Khuri, F. R., Lee, J. J., Kemp, B. L., Liu, D., Hong, W. K., and Mao, L. Hypermethylation of the death-associated protein (DAP) kinase promoter and aggressiveness in stage I nonsmall-cell lung cancer. J. Natl. Cancer Inst., *92:* 1511–1516, 2000.