Advances in Brief

Aberrant Methylation in Gastric Cancer Associated with the CpG Island Methylator Phenotype[1]

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Abstract

Aberrant methylation of 5′ CpG islands is thought to play an important role in the inactivation of tumor suppressor genes in cancer. In colorectal cancer, a group of tumors is characterized by a hypermethylator phenotype termed CpG island methylator phenotype (CIMP), which includes methylation of such genes as *p16* and *hMLH1*. To study whether CIMP is present in gastric cancer, the methylation status of five newly cloned CpG islands was examined in 56 gastric cancers using bisulfite-PCR. Simultaneous methylation of three loci or more was observed in 23 (41%) of 56 cancers, which suggests that these tumors have the hypermethylator phenotype CIMP. There was a significant concordance between CIMP and the methylation of known genes including *p16*, and *hMLH1*; methylation of *p16* was detected in 16 (70%) of 23 CIMP$^+$ tumors, 1 (8%) of 12 CIMP intermediate tumors, and 1 (5%) of 21 CIMP$^-$ tumors ($P < 0.0001$). Methylation of the *hMLH1* gene was detected in three of five tumors that showed microsatellite instability, and all three of the cases were CIMP$^+$. The CIMP phenotype is an early event in gastric cancer, being present in the normal tissue adjacent to cancer in 5 of 56 cases. These results suggest that CIMP may be one of the major pathways that contribute to tumorigenesis in gastric cancers.

Introduction

Gastric cancer is one of the most abundant neoplasms in the world (1). Genetic alterations in gastric cancer have been reported to involve the *APC*, *K-RAS*, and *p53* genes, although the frequency of these alterations is not as high as in colorectal cancer (2–4). MSI[3] is present in a subset of sporadic gastric cancer (5) and is related to molecular alterations (either mutation or methylation) in mismatch repair genes (6, 7). Alterations in the distribution of 5-methylcytosine are also important factors in multistep carcinogenesis (reviewed in Refs. 8, 9). These changes include genome-wide hypomethylation (10, 11) and hypermethylation of CpG sites in 5′ promoter regions leading to significant inhibition of gene expression. Such hypermethylation has been proposed to be an alternative way to inactivate tumor suppressor genes in cancer (8, 9). Recently, genes involved in cell cycle regulation (12–15) and mismatch repair (7, 16–19) were reported to be silenced by hypermethylation of promoter regions, and the number of genes known to be aberrantly methylated in cancer is growing.

The causes of promoter methylation in cancer remain unclear. It has previously been reported (20) that, in sporadic MSI$^+$ colorectal cancer, methylation of multiple loci was detected frequently. Recently, hypermethylation of one of the mismatch repair genes, *hMLH1*, has been shown to play a major role in causing the MSI phenotype in sporadic colorectal cancer (16–18), endometrial cancer (19), and gastric cancer (7). This hypermethylator phenotype in MSI$^+$ colorectal cancers seems to be related to a new phenotype termed CIMP (21). CIMP was identified by studying seven newly cloned CpG islands that are methylated exclusively in cancer. In colorectal cancer, CIMP$^+$ tumors include most cases with hypermethylation of known genes such as *p16*, *hMLH1*, and *THBS1*. We now report that CIMP is also present in gastric cancers, in which it is an early event. CIMP in gastric cancer also affects inactivation of known tumor suppressor genes such as *p16* and *hMLH1*. These results indicate that CIMP is one of the major pathways of tumorigenesis in gastric cancers, in which genetic alterations described to date are relatively infrequent.

Materials and Methods

Samples. Tumor specimens were obtained from 56 patients. All of the patients had given informed consent before collection of the samples according to institutional guidelines. For most of these tumors, paired normal tissues were also obtained. These tissues were frozen and stored at −80°C until DNA was extracted. MSI analysis was performed as described previously (22). Briefly, five markers, including dinucleotide and mononucleotide markers (*BAT26*, *BAT40*, *D5S346*, *D17S250*, and *MYCL1*), were amplified by PCR followed by electrophoresis. When only one marker was altered, the cases were defined as MSI-L, when 2 or more markers were altered those were defined as MSI-H and when no marker was altered those were defined as microsatellite stable. Genomic DNA was extracted using standard methods.

MSP and Bisulfite-PCR. Both MSP and bisulfite-PCR take advantage of the fact that unmethylated cytosines are efficiently converted to uracil after 16 h of Na-bisulfite treatment, whereas methylated cytosines remain unchanged. Thus, after treatment, methylated alleles have a different sequence than unmethylated alleles, which can be used to design allele-specific PCR primers (for MSP; Ref. 23). Alternatively, the primers can be designed to amplify both methylated and unmethylated alleles, with the discrimination obtained by digesting amplified fragments with restriction enzymes specific for the methylated alleles (24).

Genomic DNA was treated with Na-bisulfite as described previously (23). Briefly, 2 μg of genomic DNA were denatured by 0.2 M NaOH for 10 min at 37°C. Thirty μl of 10 mM hydroquinone (Sigma) and 520 μl of 3 M Na-bisulfite (Sigma) at pH 5.0 were added and incubated at 50°C for 16 h. Treated DNA was subsequently purified using a PCR-purification system (Promega) and precipitated with ethanol. Two μl of treated DNA were used for each PCR reaction.

The methylation status of *p16* was determined as described previously (23). Briefly, 2 μl of genomic DNA treated with Na-bisulfite was amplified using primers that specifically amplify methylated or unmethylated alleles. PCR was performed in 50-μl reaction volumes containing 1× PCR buffer [67 mM Tris-HCl (pH 8.8), 6.6 mM MgCl$_2$, 16.6 mM NH$_4$SO$_4$, and 10 mM 2-mercaptoethanol], 1.25 mM dNTP mixture, 1 μM of each primer, and 1 unit of Taq DNA polymerase (Sigma). Ten μl of PCR products were electrophoresed in 6% acrylamide gels and visualized by ethidium bromide staining.
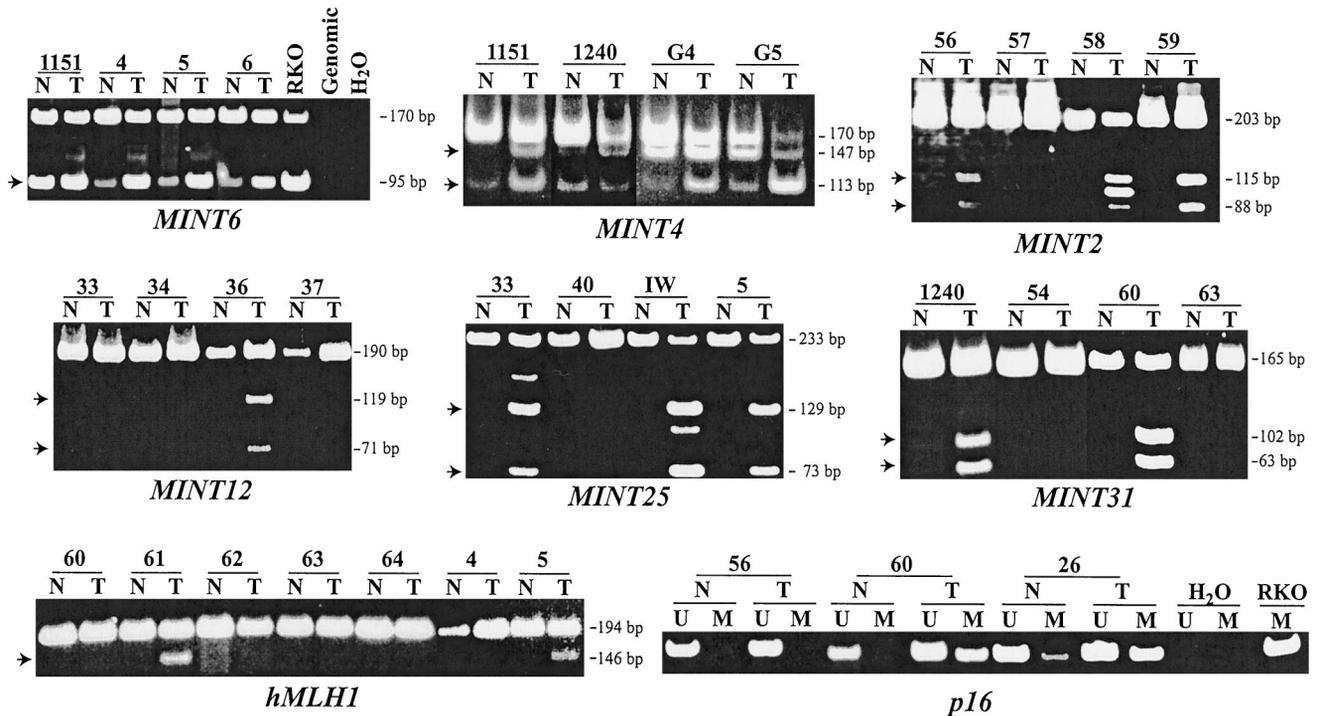
Fig. 1. Methylation analysis of multiple CpG islands in gastric cancer. Methylation of MINT2, 4, 6, 12, 25, and 31 and *hMLH1* was evaluated by bisulfite-PCR and restriction digestion. In this analysis, only methylated alleles will be digested by the restriction enzymes, and they are indicated by *arrows*. Smaller bands not indicated by arrows (*MINT2* case *58T*, *MINT25* cases *33T* and *IW*) represent nonspecific amplification products. *p16* methylation was evaluated by MSP. In this analysis, a PCR product in the *Lanes labeled M* reflects the presence of methylated alleles. Loci examined are indicated *below each gel*, and tumor numbers are indicated *above each gel*. N, normal tissue adjacent to gastric carcinoma; T, tumor DNA. RKO is a colon cancer cell line included as a positive control. All of the tumors examined were not microdissected. Therefore, in almost all of the positive cases, unmethylated alleles were also detected in the same samples, probably reflecting contamination by normal cells, although this could also represent tumor heterogeneity.

The methylation status of *hMLH1* and MINT1, 2, 4, 6, 12, 23, 25, 31, and 32 were determined by bisulfite-PCR followed by restriction digestion. Briefly, PCR primers were designed to amplify methylated alleles and unmethylated alleles equally. Two μl of DNA treated with Na-bisulfite were amplified as described previously (21).[4] Twenty to 50% of the PCR products were then digested with restriction enzymes specific to the methylated alleles by virtue of having CpG sites in their recognition sequence. After digestion, DNA was precipitated with ethanol, electrophoresed in a 6% polyacrylamide gel, and stained with ethidium bromide. The methylated alleles were evaluated by densitometry (Image Quant, Molecular Dynamics) as described previously (24).[5]

Results

To examine the methylation status of multiple loci in gastric cancer, nine sequences that fulfill the criteria for CpG islands (25) were selected for analysis. These MINT loci were originally recovered from a colorectal cancer cell line using methylated CpG island amplification, a PCR-based technique developed to clone differentially methylated DNA sequences (26). The methylation status of these nine loci was first examined for 27 gastric cancers and adjacent normal stomach mucosa by bisulfite-PCR (Fig. 1). In this analysis, DNA is first treated with Na-bisulfite for 16 h, which converts unmethylated C to U, leaving methylated C intact. After PCR amplification, the DNA is digested with restriction enzymes specific for the methylated alleles. Of the nine loci examined, four (MINT4, 6, 23, and 32) showed some degree of methylation in all of the normal stomach samples, and most of the tumors showed hypermethylation at these loci (examples in Fig. 1, summarized in Table 1). These results indicate that hypermethylation of these loci is a common event in gastric cancer. This pattern of

methylation (termed Type A for aging) is identical to that observed in colorectal cancer, in which genes affected by age-related methylation in normal colon are almost always methylated in cancers as well (21, 27). By contrast to these loci, methylation of MINT1, 2, 12, 25, and 31 was exclusively detected in gastric cancers and was absent in most normal gastric mucosa samples (examples in Fig. 1, summarized in Table 1 and Fig. 2). This pattern of methylation was also observed in colorectal cancer and was termed Type C for cancer-specific (21). The CIMP phenotype in colorectal cancer was identified using such cancer-specific clones (21). Therefore, to study CIMP in gastric cancer, the methylation status of these five cancer-specific loci was further examined in a total of 56 cases.

The frequency of methylation of the cancer-specific MINT clones varied between 34 and 71% of the 56 cases examined (examples in Fig. 1, summarized in Table 1). Of these cancer-specific clones, MINT25 stands out as the most frequently affected. This CpG island maps to chromosome 22q11–13, but the associated gene is unknown at this point. Despite its high frequency of methylation in tumors, no methylation of MINT25 was observed in either normal stomach or normal colon (21). Interestingly, MINT25 is hypermethylated in less than 10% of colorectal tumors (21), which suggests that it may play a special role in stomach neoplasia.

To establish whether there was an unusual clustering of methylation (or lack thereof), we determined the expected rates of concordance of methylation based on the measured rates of methylation at each of these five loci. If methylation was random in the 56 cases studied, we would have expected to see 2 cases with zero of five positive loci, 11 cases with one of five positive loci, 19 cases with two of five positive loci, 16 cases with three of five positive loci, 6 cases with four of five positive loci, and only 1 case in which all of the loci are methylated. The observed numbers were 9 cases with zero of five loci, 13 cases

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[4] Primer sequences and detailed cycling parameters are available at http://www.med.jhu.edu.methylation/primers.html.

[5] Detailed protocols can also be found at http://www.med.jhu.edu/methylation.

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Table 1 *Frequency of methylation of multiple loci in gastric cancer*

CIMP-I refers to CIMP-Intermediate cases.

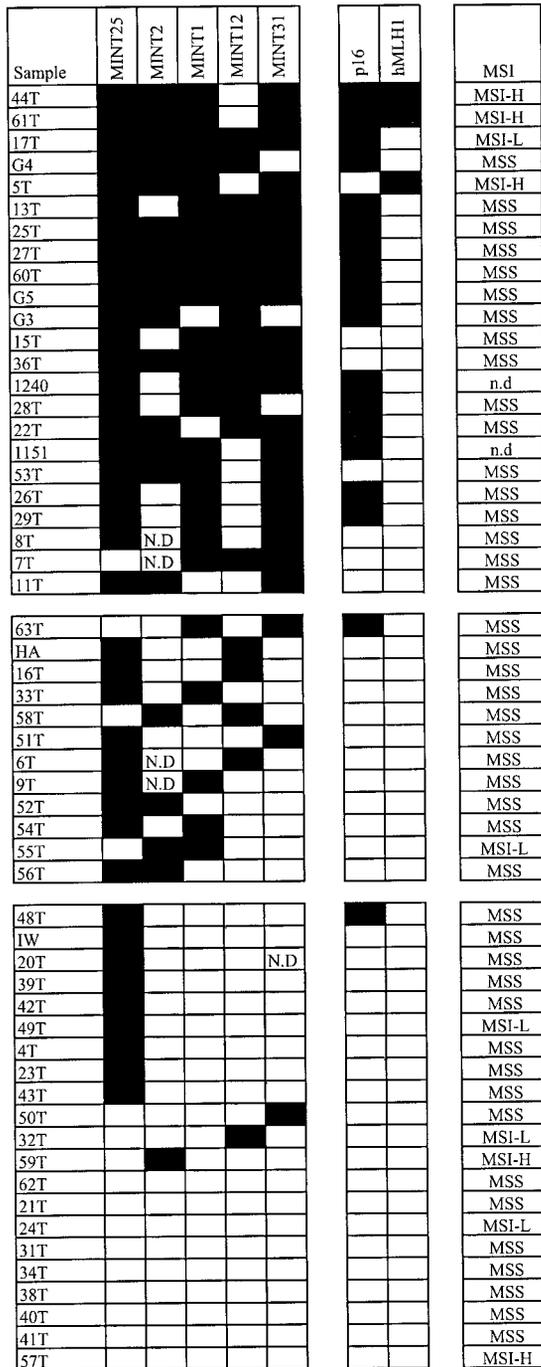| Cases	Type C (%)							Type A (%)			
	MINT1	*MINT2*	*MINT12*	*MINT25*	*MINT31*	*p16*	*hMLH1*	*MINT4*	*MINT6*	*MINT23*	*MINT32*
CIMP$^+$ (n = 23)	87	71	61	96	87	70	13	100	91	82	100
CIMP-I (n = 12)	42	40	33	75	17	8	0	100	71	71	71
CIMP$^-$ (n = 21)	0	5	5	43	5	5	0	100	67	56	67
Total[a] (n = 56)	45	38	34	71	42	32	5	100	78	70	74

[a] n = 27 for Type A loci.

Fig. 2. CIMP in gastric cancer. The methylation status of 7 CpG islands in 56 gastric cancers is shown. *Each column* represents a separate gene locus indicated *on the top*. *Each row* represents a primary gastric cancer. *Black squares*, methylated alleles in the tumor. *White squares*, unmethylated alleles in the tumor. *N.D* and *n.d*, not done. *The top group* is CIMP$^+$ (=three MINT loci methylated); *the bottom group* is CIMP$^-$ (<two MINT loci methylated); and *the middle group* is CIMP-I (intermediate, 2 MINT loci methylated).

with one of five loci, 11 cases with two of five loci, 7 cases with three of five loci, 10 cases with four of five loci, and 6 cases with five of five loci methylated, respectively, which was significantly different from what was expected ($P = 0.004$ by χ^2 analysis performed by dividing the cases into three groups with zero of one, two of three, and four of five loci methylated). As shown in Fig. 2, this difference is due to an excess of cases with high levels of methylation, as well as cases with no methylation at all. This indicates the presence of a hypermethylator phenotype in gastric cancer similar to that seen in colorectal cancer and referred to as CIMP (21). The cases showing methylation at more than three loci were defined as CIMP$^+$, those methylated at two loci called CIMP-I (intermediate), and the cases where less than two loci were methylated were defined as CIMP$^-$. The above analyses and classification remain largely unchanged if we exclude MINT25. In fact, in the absence of MINT25, the distinction between the three CIMP groups becomes more pronounced.

To investigate whether CIMP affects the methylation of known genes in gastric cancers, the cyclin-dependent kinase inhibitor *p16* on chromosome 9p21 (12–14) and the mismatch repair gene *hMLH1* on chromosome 3p21 (7, 16–19), which are known to be methylated in various cancers, were examined. Of 56 cases, 18 (32%) showed methylation of *p16*, and 3 (5%) showed methylation of *hMLH1*. Just like the MINT loci examined, methylation of *p16* and *hMLH1* clustered in CIMP$^+$ cases (Fig. 2). *p16* was methylated in 16 (70%) of 23 CIMP$^+$ tumors, 1 (8%) of 12 CIMP-I tumors, and 1 (5%) of 21 CIMP$^-$ tumors ($P < 0.0001$ by Fisher's exact test). All three of the gastric cancers that showed *hMLH1* methylation were CIMP$^+$. Next, we examined the relation between MSI status and CIMP. In total, 5 tumors were MSI-H, 4 tumors were MSI-L, and 47 tumors were microsatellite stable. Three (60%) of five MSI-H tumors were methylated at *hMLH1*, and all three of the cases were CIMP$^+$. None of the other tumors had *hMLH1* methylation. The other two MSI-H tumors were CIMP$^-$.

Gastric cancer often arises from a background of dysplasia (28). To determine whether CIMP could precede cancer formation, we next examined the methylation status of multiple CpG islands in normal stomach mucosa adjacent to cancer. Although the methylation of cancer-specific loci was rarely detected in normal stomach mucosa, in five cases (Cases 17, 44, 60, 28, and 1151), the normal mucosa adjacent to CIMP$^+$ tumors showed methylation of multiple loci, including *p16* (examples in Fig. 3). These data suggested the possibility that some CIMP$^+$ gastric cancers arise from CIMP$^+$ dysplasia. Interestingly, in one case (No. 44), the apparently normal CIMP$^+$ tissue was unmethylated at *hMLH1*, whereas the adjacent cancer was in fact methylated at this locus (data not shown).

Finally, the clinicopathological features of these tumors were studied in reference to the CIMP phenotype. There was no statistical difference between CIMP$^+$ and CIMP$^-$ cases in age (64 ± 2.6 *versus* 64.7 ± 2.4), tumor size, gender, or presence of lymph node metastasis. However, there seemed to be a significant difference in the stages of the tumors. Six of 23 CIMP$^+$ tumors were early stage (Stage I),
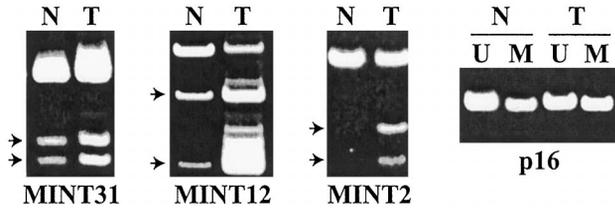
Fig. 3. Hypermethylation of multiple CpG islands in normal stomach mucosa adjacent to a CIMP$^+$ gastric cancer (Case 17). Methylation was analyzed as in Fig. 1. Loci examined are indicated *below each panel*. Aberrant methylation was detected for MINT2, 12, and 31 and *p16* in gastric cancer and for MINT12 and 31 and *p16* in adjacent normal stomach mucosa.

whereas 0 of 32 CIMP$^-$ or CIMP-I tumors were early stage ($P < 0.01$ by Fisher's exact test).

Discussion

In this report, we demonstrate that about one-half of all gastric cancers have concordant methylation of multiple loci that seem to be methylated *de novo* during cancer progression. We suggest that these tumors have a hypermethylator phenotype (CIMP), which leads to the simultaneous inactivation of multiple genes. In gastric cancer, CIMP also affects the methylation of known genes, including *p16* and *hMLH1*. As in colorectal cancer, we suggest that CIMP is an early event in gastric cancer that leads to cancer formation and progression through the silencing of tumor suppressor genes. It also seems that CIMP followed by *hMLH1* methylation may explain many cases of gastric cancer with MSI. The high frequency of the CIMP phenotype may also explain why genetic lesions are relatively rare in gastric cancer. This model of cancer progression through a hypermethylator phenotype may be applicable to other human neoplasms. In preliminary data, we have also observed CIMP in endometrial cancers and acute leukemias, and other studies have revealed the presence of a hypermethylator phenotype in bladder cancers (29), prostate cancers (29), and breast cancers (30).

The mechanism of this multiple CpG island methylation remains unclear. CIMP affects only a subset of tumors and a limited number of genes. The defect that leads to CIMP could be either aberrant *de novo* methylation (through a mutation in DNA-methyltransferase for example) or loss of protection against *de novo* methylation through the loss of a *trans*-activating factor (31–33). These CIMP$^+$ tumors may then develop through a pathway that heavily relies on this methylation defect, whereas others rarely show tumor suppressor gene methylation. An important question is whether the concordant methylation described here provides a growth advantage to affected cells or whether it just accompanies tumor development. Aberrant methylation often occurs in CpG islands outside of promoter regions (29) in which it may not affect gene transcription. Additional studies are necessary to clarify whether the genes methylated in cancer simply reflect the genome wide methylation defect or whether stochastic methylation of each CpG island results from selective pressures.

On the basis of a limited number of cases, we found that CIMP$^+$ gastric cancers had a relatively earlier stage when compared with CIMP$^-$ tumors. It is somewhat surprising that six of six nonadvanced gastric cancers showed hypermethylation of multiple loci. Furthermore, CIMP was also detected in some cases in normal mucosa adjacent to cancer, where dysplasia is a common feature. These results indicate that methylation of the genes examined may not simply accumulate during tumor progression because they are very early events in some cases. Furthermore, it is possible that CIMP$^-$ tumors that probably evolve along more classical genetic pathways may progress rapidly and are, therefore, rarely found at early stages. Interestingly, MSI$^+$ colorectal cancer, which are often CIMP$^+$, have a better prognosis than MSI$^-$ colorectal cancer (34). Additional studies are necessary to clarify the detailed clinicopathological features of tumors with and without CIMP using a large number of cases.

The methylation profiles that we show here may also be useful as potential diagnostic markers. Loss of heterozygosity is difficult to evaluate in gastric cancer because of the substantial amount of normal cell component in a subset of tumor tissues (35). Detection of methylation can be done as a gain of signals unlike detecting loss of heterozygosity. Therefore, these loci may serve as good markers to detect tumor cells from biopsies, serum, gastric lavage, and so forth. Some tumor suppressor genes such as *p16* have already been shown to be useful for such a purpose (36). Here, we show a high frequency of methylation of MINT25 in gastric cancer, and this gene is rarely methylated in colorectal, lung, prostate, and brain tumors (data not shown). This CpG island may then be useful as a specific diagnostic marker in gastric cancer in which there is no perfect marker for noninvasive diagnosis. It may also be useful to combine some of these markers to detect circulating tumor cells in blood or predict the prognosis of patients. Moreover, methylation profiling may be useful to establish the epigenotype of each tumor to detect potential differences in sensitivity to chemotherapy, occurrence of metastasis, and overall prognosis.

Acknowledgments

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