Rapid Identification of Promoter Hypermethylation in Hepatocellular Carcinoma by Pyrosequencing of Etiologically Homogeneous Sample Pools

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Aberrant DNA methylation patterns have been identified in a variety of human diseases, particularly cancer. Pyrosequencing has evolved in recent years as a sensitive and accurate method for the analysis and quantification of the degree of DNA methylation in specific target regions. However, the number of candidate genes that can be analyzed in clinical specimens is often restricted by the limited amount of sample available. Here, we present a novel screening approach that enables the rapid identification of differentially methylated regions such as promoters by pyrosequencing of etiologically homogeneous sample pools after bisulfite treatment. We exemplify its use by the analysis of five genes (*CDKN2A*, *GSTP1*, *MLH1*, *IGF2*, and *CTNNB1*) involved in the pathogenesis of human hepatocellular carcinoma using pools stratified for different parameters of clinical importance. Results were confirmed by the individual analysis of the samples. The screening identified all genes displaying differential methylation successfully, and no false positives occurred. Quantitative comparison of the pools and the samples in the pool analyzed individually showed a deviation of ~1.5%, making the method ideally suited for the identification of diagnostic markers based on DNA methylation while saving precious DNA material. (*J Mol Diagn* 2007, 9:510–520; DOI: 10.2353/jmoldx.2007.060209)

DNA methylation occurring at the 5-position of cytosines in the context of the dinucleotide CpG is of particular importance for proper development and gene regulation and is strongly implicated in the pathogenesis of various diseases.¹ Epigenetic deregulation, ie, aberrant DNA methylation levels, altered patterns of histone tail modifi-

cations, and chromatin structure, has now been recognized as a hallmark of cancer.^{2–4} An overall decrease in DNA methylation (global hypomethylation) is accompanied by a region- and gene-specific hypermethylation of CpG islands. Genes involved in DNA repair, detoxification, cell-cycle regulation, and apoptosis are thereby often inappropriately inactivated. Epigenetic changes occur early in the progression process and often precede malignancy, and epigenetic lesions in normal tissue might set the stage for neoplasia.⁵ Methylation patterns can be shared by different types of tumors as well as being tumor type-specific.⁶ Analysis of DNA methylation patterns has proven useful as biomarker for the early diagnosis, classification, prognosis, and therapy of human cancers.^{7–9} Realizing the important participation of DNA methylation in the pathogenesis of cancer and other diseases, a variety of techniques for the study of DNA methylation has been developed in the last few years.^{2,7,8}

One of the methods that has recently received much attention for the simultaneous analysis and quantification of the degree of methylation at several CpG positions in close proximity is pyrosequencing.^{10–13} The pyrosequencing technology is based on the luminometric detection of pyrophosphate that is released on nucleotide incorporation and converted into a light signal by a cascade consisting of four enzymes.¹⁴ Its ease-of-use, high reliability, and flexibility have made pyrosequencing an analysis platform that has been widely used for various diagnostic applications such as routine (multiplex) genotyping,^{15,16} bacterial typing,^{17–19} and sensitive detection of mutations.²⁰ One of its major strengths is the quantitative nature of the results. The bioluminometric response is linear ($R^2 > 0.99$) for the sequential addition of up to five identical nucleotides (C, G, and T) or three α -S-

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dATPs. Pyrosequencing has therefore been used as a method to determine allele frequencies of SNPs in pools of samples. Deviations from results obtained by genotyping of individual samples were as low as 1.6% (determined from peak height) in a pool of 1126 samples, and the technology was found to be able to detect differences in allele frequencies of less than 2% between pools of DNA.^{21–23}

Pyrosequencing is ideally suited for DNA methylation analysis after bisulfite treatment of DNA because it combines the ability of direct quantitative sequencing, reproducibility, speed, and ease-of-use and is becoming more and more used. Besides the identification of genes aberrantly silenced by promoter hypermethylation in cancer,^{24,25} pyrosequencing has been used as a reference method for validation of newly developed methods for DNA methylation analysis,²⁶ to monitor chemically induced demethylation in leukemia patients,²⁷ as well as for a diagnostic test for aberrant methylation in the imprinting disorders Prader-Willi and Angelman syndromes.²⁸ One of the major limitations for the identification of novel genes involved in tumorigenesis is the amount of available sample that is in most cases derived from primary tumors. The successive use of several sequencing primers on the same DNA template (serial pyrosequencing) significantly reduces cost, labor, and analysis time as well as saving precious DNA samples for the analysis of a specific region amplified in a single polymerase chain reaction (PCR).²⁹ However, it still requires prior knowledge of the presence of epimutations in the target. Using a candidate gene approach, we found in various projects that only 5 to 10% of genes hypothesized to display aberrant methylation actually show differential methylation in a certain tumor type (J.T., unpublished data). Here, we present a novel method that enables rapid screening for differential methylation using etiologically homogeneous pools of samples. We analyzed quantitatively the DNA methylation levels at 112 CpGs in the promoter or differentially methylated region (DMR) of five genes that have previously been found to be implicated in the pathogenesis of hepatocellular carcinoma (HCC): the cell-cycle regulator *CDKN2A* (*p16*),³⁰ glutathione S-transferase π (*GSTP1*) involved in detoxification and drug resistance,³¹ the insulin-like growth factor 2 (*IGF2*),³² the DNA mismatch repair gene *MLH1*,³³ and β -catenin (*CTNNB1*), which is mutated in 26 to 40% of human HCCs and not regulated by methylation of its promoter.^{34,35} Samples were grouped into pools after bisulfite treatment depending on the etiology (hepatitis B or C virus infection and alcohol consumption) and on the tumoral and nontumoral tissue status: control liver, non-cirrhotic or cirrhotic adjacent nontumoral liver, hepatic adenoma, and HCC with and without β -catenin mutation. All genes displaying variable methylation patterns were successfully detected in the pools, and no false-negative results occurred. The high quantitative concordance of the data accumulated by single sample analysis compared with the pooled sample amplification clearly demonstrates the ability of our method to preserve precious sample resources from clinical specimens without loss of accuracy. Our method therefore provides the possibility

to screen a large number of potential target genes even with a limited amount of sample.

Materials and Methods

Samples

Tumor samples were collected in accordance with French law and ethical guidelines, and their use was approved by the institutional ethics committee. Operative notes and pathology reports were subjected to exhaustive review. Samples used in this study were 27 HCCs and three hepatic adenomas and their nontumoral counterparts and 10 normal control livers without cirrhosis. DNA from peritumoral liver tissue, a hepatic adenoma, and the carcinoma was available from one patient. The degree of hepatic fibrosis in noncancerous liver was graded according to the METAVIR classification.³⁶ Seventeen HCCs developed on extensive fibrosis or cirrhosis (F3 and F4). Etiologies of the tumors were hepatitis C virus (HCV) infection for 11 cases (41%), hepatitis B virus (HBV) infection for four cases (15%), and alcohol consumption for seven cases (26%). Five cases (18%) developed in the absence of known etiology. Fourteen HCC and the three hepatic adenomas displayed β -catenin mutations after RNA isolation and further sequencing with the primers F1/R1, F1/R2, and F2/R2, as described previously.³⁵

Preparation of DNAs

DNAs were extracted from frozen tissue samples using standard methods, and DNA concentrations of extracted DNA were determined using the Quant-iT dsDNA broad range assay kit (Invitrogen, Cergy Pontoise, France) on a SpectraMAX Gemini XPS microplate spectrofluorometer (Molecular Devices, St. Grégoire, France) and normalized to a concentration of 50 ng/ μ l. Unmethylated DNA was obtained by whole genome amplification of a DNA extracted from a lymphoblastoid cell line from the CEPH/Utah collection using the REPLI-g kit (Qiagen, Courtaboeuf, France) using 50 ng as input DNA.

Highly methylated DNA was obtained by treating genomic DNA of the same lymphoblastoid cell line with the methylase SssI (Ozyme, St. Quentin-en-Yvelines, France). In brief, 7.5 μ l of NE-buffer 2, 10 nmol of S-adenosylmethionine (SAM), and 6 U of SssI were added to 4.5 μ g of human genomic DNA in a final volume of 67.5 μ l. The solution was incubated at 37°C in a water bath. After 3 hours and again after an additional 2 hours, 10 nmol of SAM and 6 U of SssI were added, and the reaction was incubated overnight at 37°C. The enzyme was inactivated at 95°C for 5 minutes and DNA stored at –20°C until further use.

Bisulfite Treatment and Adjustment of Concentration

One μ g of DNA was bisulfite converted using the MethylEasy HT kit for Centrifuge (Human Genetic Signatures,

Table 1. Sequences of Primers Used for Amplification and Pyrosequencing Reactions, Including Genbank Accession Numbers and Nucleotides (Nt) Corresponding to the Amplified Fragments as Well as the Annealing Temperatures for the Respective PCR Amplifications

Gene	PCR primer forward	PCR primer reverse	Temperature PCR (°C)
<i>CDKN2A</i> (262 bp) AF527803 Nt 19893 to 20154	5'-GAGGGGTTGGTTGGTTATTAGA-3'	5'-Biotin-TACAAACCCTCTACCCACCTAAAT-3'	64
<i>CTNNB1</i> (294 bp) AY463360 Nt 1786 to 2079	5'-TGGGGTGTCTTAGGTATTTTATTT-3'	5'-Biotin-TAAACTACTCTCAAACCTTCCTC-3'	64.2
<i>GSTP1</i> (301 bp) AY324387 Nt 1670 to 1970	5'-GAAAGAGGGAAAGGTTTTTT-3'	5'-Biotin-CCATACTAAAACTCTAAACCCCATC-3'	58
<i>GSTP1</i> (301 bp) AY324387 Nt 1670 to 1970	5'-Biotin-GAAAGAGGGAAAGGTTTTTT-3'	5'-CCATACTAAAACTCTAAACCCCATC-3'	58
<i>GSTP1</i> (212 bp) AY324387 Nt 1845 to 2056	5'-GGGATTATTTTTTATAAGGTT-3'	5'-Biotin-TCCTAAATCCCTTAAACCCC-3'	54
<i>MLH1</i> (297 bp) AY217549 Nt 1504 to 1800	5'-GGGAGGTTATAAGAGTAGGGTTAA-3'	5'-Biotin-TCTCAACTCTATAAATTACTAAATCTCTTC-3'	61.4
<i>IGF2</i> DMR2 (255 bp) AF125183 Nt 7881 to 8100	5'-GGGAAAGGGGTTTAGGATTTTAT-3'	5'-Biotin-ATAATTTACTCCCCCTTCAACCTC-3'	60

CpGs are numbered in the order of appearance from the 5' end of an amplification product. Y, pyrimidine.

(table continues)

North Ryde, NSW, Australia) according to the manufacturer's instructions. Concentration of the bisulfite converted DNAs was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and normalized to a concentration of 20 ng/ μ l. Pools were constructed from bisulfite-converted DNAs.

DNA Methylation Analysis by Pyrosequencing

Quantitative DNA methylation analysis of the bisulfite-treated DNA was performed by pyrosequencing or, in case of several sequencing primers, by serial pyrosequencing.²⁹ Primers for PCR amplification and pyrosequencing were purchased from Biotex (Buch, Germany). Regions of interest were amplified using 20 ng of bisulfite-treated human genomic DNA and 5 to 7.5 pmol of forward and reverse primer, one of them being biotinylated. Sequences of the oligonucleotides for PCR amplification and pyrosequencing are given in Table 1. Reaction conditions were 1 $\times$ HotStar Taq buffer supplemented with 1.6 mmol/L MgCl₂, 200 mmol/L dNTPs, and 2.0 U of HotStar Taq polymerase (Qiagen) in a 25- μ l volume. The PCR program consisted of a denaturing step of 15 minutes at 95°C followed by 50 cycles of 30 seconds at 95°C, 30 seconds at the respective annealing temperature (Table 1), and 20 seconds at 72°C, with a final extension of 5 minutes at 72°C. Amplification products were purified and rendered single-stranded on a Pyrosequencing workstation (Pyrosequencing AB, Uppsala, Sweden). PCR products were incubated for 10 min-

utes at room temperature with 51 μ l of binding buffer (10 mmol/L Tris, 2 mol/L NaCl, 1 mmol/L ethylenediamine tetraacetic acid, and 0.1% Tween 20, pH 7.6, adjusted with 1 mol/L HCl) and 4 μ l of streptavidin-coated Sepharose beads (GE Health Care, Uppsala, Sweden). The binding mix was aspirated, and the template was successively washed with 70% ethanol, rendered single-stranded with 0.2 mol/L NaOH, and neutralized with washing buffer (10 mmol/L Tris, pH 7.6, adjusted with 4 mol/L acetic acid). Beads were released into 40 μ l of annealing buffer (20 mmol/L Tris and 2 mmol/L magnesium acetate, pH 7.6, adjusted with 4 mol/L acetic acid) containing 15 pmol of the respective sequencing primer (Table 1). Primers were annealed to the target by incubation at 80°C for 2 minutes. Quantitative DNA methylation analysis was performed on a PSQ 96MA system with the PyroGold SQA reagent kit (Pyrosequencing), and results were analyzed using the Q-CpG software (V1.0.9; Pyrosequencing). Stripping of the template strand for subsequent annealing of a new sequencing primer (serial pyrosequencing) was performed by adding 20 μ l of binding buffer to the completed sequencing reaction and resuspending the Sepharose beads. The binding mix was then purified without further incubation and the biotinylated template strand rendered again single-stranded using the above-described purification protocol. This process completely removes all DNA strands that have been *de novo* synthesized during the last sequencing run as well as remaining sequencing primers.²⁹

Table 1. *Continued*

Pyrosequencing primer	CpG number	CpGs
5'-TGTTATTAGAGGGTG-3'	1 to 7	7
5'-GAGGGGAGAGTAGGTAG-3'	8 to 13	6
5'-GGGGAGTAGTATGGAGTTT-3'	14 to 21	8
5'-GGGTGAGGTAGAGGA-3'	20 to 28	9
5'-CGGTAGTAGATTGTTGGG-3'	4 to 20	17
5'-GTTGYGTYGGTGG-3'	21 to 27	7
5'-TTTYGGTTTGTGGTAGTA-3'	28 to 34	7
5'-GTAGTTTTTYGTTATTAGTGA-3'	33 to 38	6
5'-GGATTATTTTATAAGGT-3'	25 to 32	8
5'-CGAACCTTATAAAAATAATC-3'	17 to 11	7
5'-GTAGTTTTTYGTTATTAGTGA-3'	9 to 14	6
5'-GAGAGGAGGAGTTTGAGA-3'	3 to 7	5
5'-TTTTTATTGGTTGGATATTT-3'	8 to 18	8
5'-AAAACGAATTAATAGGAAGA-3'	11 to 18	11
5'-GATTTTATYGGAAAGTA-3'	2 to 6	5
5'-GGGTGATAYGTTTTTTT-3'	6 to 13	8
5'-TTTTATAGTATAGAGAGGT-3'	16 to 18	3

Statistics

Quantitative DNA methylation values are represented by boxplots in which the distribution of data points in a sample set is displayed without any assumption of statistical distribution. Lower and upper quartiles of the data delimit the box, the median is represented by the bold black dot in the box, and minimal and maximum values are indicated by the lines. A Mann-Whitney *U*-test was used to compare quantitative methylation values between two groups. For *IGF2*, the average of all samples was calculated, and for each sample, the absolute distance of the methylation value from this average was used to define values for ranking because both hypo- and hypermethylation occur in the tumors.

Results

The rationale behind this study was to evaluate pyrosequencing-based DNA methylation analysis in pools of samples that are homogenized for distinct etiologies to enable rapid and cost-effective screening of multiple genes potentially displaying differential methylation in a candidate region. As a proof-of-principle, we chose to analyze the methylation patterns in the promoter regions of four genes (*CDKN2A*, *GSTP1*, *MLH1*, and *CTNNB1*) and the differentially methylated region 2 (DMR2) of *IGF2* in a panel of 71 samples (10 control livers, 27 paired HCC samples, three paired adenomas, and two additional peritumoral livers). Aberrant methylation in hepatocellular carcinogenesis had previously been reported for

CDKN2A, *GSTP1*, *MLH1*, and *IGF2*, but not for *CTNNB1*, which is mutated in a subset of HCCs.

Limit of Detection

We and others^{12,21} have previously shown that pyrosequencing-based DNA analysis has a limit of detection of ~3% for the minor component of a quantitative signal and a quantitative resolution of 5% or better. However, because this parameter is of utmost importance for the approach, we chose to reverify the limit of detection using the *CDKN2A* amplicon as a model system. Completely *in vitro* methylated and unmethylated DNAs were bisulfite-treated and normalized to a concentration of 20 ng/ μ l using a NanoDrop spectrophotometer. The use of the more accurate fluorescent dyes as used in the Quant-iT kit is no longer possible because these are highly selective for double-stranded DNA, and strands are no longer complementary after bisulfite treatment. The maximal error in the determination of the concentration after normalization because of pipetting and other random fluctuations was estimated to be 20 ± 1.3 ng/ μ l (6.5%). Completely methylated DNA was diluted into the unmethylated DNA to create mixtures with a methylation degree of 0, 2, 5, and 10%. Figure 1 confirms the limit of detection being at 2% and clearly demonstrates the ability of our approach to detect methylation differences as low as 2 to 5%. We therefore decided to divide the samples into pools consisting of a maximum of eight samples. This approach permits the identification of aberrant methylation if a single sample displays a methylation degree of

Figure 1. Pyrograms obtained by the analysis of mixtures with a known degree of methylation in the promoter of *CDKN2A* with 0% (A), 2% (B), 5% (C), and 10% (D) of methylation. E: The linearity of the signal for the sixth CpG position shown in A–D is demonstrated. Quantitative differences as low as 2 to 5% can be detected by pyrosequencing. Similar linear regression coefficients were obtained for the other five CpGs. CpG 1: $y = 1.3x + 2.3$, $R^2 = 0.998$; CpG 2: $y = 1.57x + 2.3$, $R^2 = 0.997$; CpG 3: $y = 1.20x + 1.0$, $R^2 = 0.984$; CpG 4: $y = 0.88x + 6.6$, $R^2 = 0.978$; CpG 5: $y = 0.79x + 4.2$, $R^2 = 0.998$. F: The linearity of the signal throughout the entire dynamic range (0 to 100%) for the sixth CpG position is shown. All assays were performed in triplicate. The resolution and linear correlation is similar for *IGF2* as demonstrated in G.

20 to 25% against a background of seven unmethylated samples. If more than one sample is methylated, methylation levels of ~10% are sufficient to be detected.

To assess the variation induced by bisulfite treatment, two independent bisulfite treatments were performed, and quantitative DNA methylation levels were analyzed at the *CDKN2A* promoter. Methylation degrees at individual CpG positions between showed a high degree of reproducibility with a mean variation of 1.9% (range, -5.9 to $+6.3$ for individual CpG positions). This variability is in the same range as the variation observed in independent PCR amplifications. These results are in good concordance with recently published results demonstrating a SD of 3.5% between seven independent bisulfite treatments.³⁷ Bisulfite treatment does therefore not have a major influence on quantitative methylation values if well standardized.

Construction of Pools

For proof-of-principle, samples were divided into 12 pools. Seven of the 10 normal liver tissues were grouped together into one pool and the six available peritumoral liver samples without known etiology into the second pool. A third pool was constructed from peritumoral non-cirrhotic livers from alcoholic individuals ($n = 3$), and the fourth pool was built from DNA extracted from peritumoral noncirrhotic liver from HBV- or HCV-infected individuals ($n = 4$). Peritumoral cirrhotic liver tissues were divided into three pools, one for patients with increased alcohol consumption ($n = 5$) and two pools for cirrhotic peritumoral liver tissues from patients with HBV or HCV infec-

tion ($n = 6$ each). The three hepatic adenomas were pooled together for further analysis. HCCs were divided into four pools: one for HCCs without β -catenin mutations from HBV- or HCV-infected individuals ($n = 7$); a second pool of HCCs carrying no mutations in β -catenin regrouping carcinoma developed on alcohol consumption or no known etiology ($n = 6$); a third for HCCs with β -catenin mutations from HBV- or HCV-infected individuals ($n = 8$); and the fourth consisting of HCCs with β -catenin mutations regrouping carcinoma developed on alcohol consumption or no known etiology ($n = 6$).

Analysis of Methylation Pattern in Pools

Methylation patterns were analyzed at 112 CpGs in the four promoter regions and the DMR in all 10 pools (Table 2). These results clearly provide evidence for variable methylation patterns between pools for *CDKN2A*, *GSTP1*, and *IGF2*, whereas all pools are consistently unmethylated for *MLH1* and as expected for *CTNNB1*. Based on this analysis only the three variable regions would be carried on to further analysis of all individual samples, whereas *MLH1* and *CTNNB1* would not be further investigated. It should be pointed out that no conclusion about methylation prevalence and distribution in a distinct sample group can be drawn from the information obtained by the analysis of the pools except the presence or absence of variable methylation patterns between pools. This is exemplified by the difference that is found between pools with identical tumor status and etiology such as the two pools constructed from peritumoral cirrhotic liver from HBV- or HCV-infected individuals for *GSTP1* and *IGF2*.

Analysis of Individual Samples

To demonstrate the reliability of our approach, we chose to analyze the entire sample cohort in all five genes. As expected from the analysis of the pools all samples were unmethylated for *MLH1* and *CTNNB1*, whereas methylation patterns varied in *IGF2*, *CDKN2A*, and *GSTP1*. Figure 2 shows boxplot diagrams for the five genes and the level of statistical significance for the different distributions. Normal liver samples and peritumoral liver samples without cirrhosis were grouped together for easier visualization because no difference between these groups was found in any of the analyzed genes. *CDKN2A* methylation was found in none of the normal—noncirrhotic—livers, none of the three tested adenomas, and in one of the cirrhotic livers (6%). However, methylation was detected in 7 of 13 (54%) of human HCCs and in 11 of 14 (79%) β -catenin-mutated HCCs with a very significant difference in the quantitative methylation distribution between the two groups of HCCs. Significant differences were also observed between all groups of normal and the cancerous samples. The analysis of promoter methylation of *GSTP1* revealed that 22 of 25 (88%) normal and peritumoral livers and 11 of 14 (69%) cirrhotic livers had a significant level of methylation, and all (100%) of the cancerous samples including the adenomas were found to be methylated. Quantitative methylation differences

Table 2. Average DNA Methylation Values in Percentage for the Analysis of the Five Genes in the 10 Pools

	Number of samples in pool	<i>CTNNB1</i>	<i>MLH1</i>	<i>GSTP1</i>	<i>CDKN2A</i>	<i>IGF2</i>
Number of CpG positions analyzed		30	16	21	28	17
Known etiology						
Normal control liver	7	2.5	3.1	22.7	4.5	51.1
Peritumoral noncirrhotic liver without known etiology	5	3.2	2.3	13.5	3.9	50.4
Peritumoral noncirrhotic liver from HBV- or HCV- infected individuals	4	2.4	3.1	10.1	4.6	45.2
Peritumoral noncirrhotic liver from alcoholic individuals	3	2.2	3.4	14.9	4.6	50.8
Peritumoral cirrhotic liver from alcoholic individuals	5	3.2	2.4	19.8	4.8	46.6
Peritumoral cirrhotic liver from HBV- or HCV-infected individuals (pool 1)	6	2.1	2.4	15.8	4.1	49.1
Peritumoral cirrhotic liver from HBV- or HCV-infected individuals (pool 2)	6	2.5	3.3	7.1	4.5	40.3
Hepatic adenoma with β -catenin mutations	3	2.7	3.5	29.7	3.0	49.1
HCC without β -catenin mutations from HBV/HCV- infected individuals	7	2.1	3.2	37.0	14.8	38.5
HCC without β -catenin mutations from alcoholic individuals or developed without known etiology	6	2.4	3.8	32.0	16.8	38.5
HCC with β -catenin mutations from HBV/HCV- infected individuals	8	2.2	3.3	47.0	32.8	42.4
HCC with β -catenin mutations from alcoholic individuals or developed without known etiology	6	2.4	3.4	33.5	24.6	44.2

Noncancerous liver was considered as fibrotic or cirrhotic when graded as F3 or F4 according to the METAVIR classification.

were highly significant between normal samples and cancerous samples, and also the comparison of the quantitative distribution showed a significant difference between the β -catenin adenomas and the mutated HCCs and also between mutated and nonmutated HCCs. To exclude a technical problem, we amplified the target region around the transcription start of *GSTP1* with a second independent primer set (Table 1). The presence of methylation in the normal samples was confirmed, and quantitative measures for the analyzed CpG positions varied on average by 2.6% (range, -3.08 to $+7.46\%$) between the two primer sets. In addition, a recent publication by another research group used a very similar primer set for amplification as that used by us and found it well suited for methylation analysis.³⁸ The region ana-

lyzed in *IGF2* is a differentially methylated region displaying allelic-specific methylation. In the normal livers an average methylation of 46.8% was found. Cirrhotic livers showed a slight but consistent and statistically significant decrease in methylation of $\sim 5\%$. For the HCC samples, two phenomena occur simultaneously. Eight of 13 HCC (61%) showed a marked hypomethylation of the entire region, and three samples (23%) showed a trend toward hypermethylation. Methylation values of β -catenin-mutated HCCs showed a tendency toward more extreme values for the hypomethylated (43%) as well as for hypermethylated (21%) samples compared with the non-mutated HCCs, but this did not reach the level of statistical significance.

Comparison between Measure of Individual Samples and Pools

The negative results for *CTNNB1* and *MLH1* were successfully predicted by the analysis of the pools, and the variable DNA methylation levels between the different etiological groups were estimated with high precision for *CDKN2A*, *GSTP1*, and *IGF2*. Tables 3 and 4 give two examples for the analysis of DNA methylation patterns for a pyrosequencing primer in *GSTP1* for the normal liver pool with methylation content of 23.7% averaged over eight CpGs and for a primer in *IGF2* analyzing a HCC pool developed on alcohol consumption or without a known etiology. Comparison of the results obtained by the analysis of the pooled DNA sample to the average of the individual samples comprised in the pool show an average deviation of 1.4% (range, -2.2 to 1.1%) for *GSTP1* CpGs 25 to 32 and 1.3% (range, -2.3 to 2.6%) for CpGs 6 to 15 of the *IGF2* DMR2. The slight but consistent drop of methylation levels in normal livers compared with

Figure 2. Graphical representation (boxplots) of the results obtained by the analysis of the individual samples for 112 CpGs in five genes (*CDKN2A*, *GSTP1*, *MLH1*, *IGF2*, and *CTNNB1*) and the level of statistical significance for differential methylation values. * $P < 0.05$, ** $P < 0.005$, and *** $P < 0.0005$.

Table 3. Quantitative DNA Methylation Values for a Pyrosequencing Primer Analyzing Eight CpGs in the Promoter of *GSTP1* in the Pool of Normal Livers and Analysis of the Seven Samples Comprised in the Pool Individually

Sample	CpG 25	CpG 26	CpG 27	CpG 28	CpG 29	CpG 30	CpG 31	CpG 32
Pool normal liver	28.6	32.0	20.2	33.2	36.2	32.3	40.2	44.3
N 1	13.5	16.3	11.6	18.2	17.6	15.2	18.7	28.7
N 2	29.4	38.1	22.5	42.3	43.3	36.6	42.8	47.6
N 3	32.3	31.9	18.7	42.4	40.0	38.7	45.7	46.9
N 4	32.2	38.3	23.4	34.3	35.9	39.7	46.2	48.2
N 5	30.3	34.1	23.0	39.0	37.9	37.9	42.7	43.9
N 6	17.9	26.3	13.6	24.4	30.3	26.4	31.5	35.7
N 7	29.3	34.8	19.4	39.6	38.3	37.7	41.9	44.3
Average of individual samples	26.4	31.4	18.9	34.3	34.7	33.2	38.5	42.2
Δ Pool/individual samples	-2.2	-0.6	-1.3	+1.1	-1.5	+0.9	-1.7	-2.1

Values are given in percentage, and the average values of the individual samples are compared with the values obtained in the pool.

cirrhotic livers in the *IGF2* DMR2 is another example of already successfully detected changes in the pools (Table 2). Comparison with the individual samples also explained perfectly the differences between the two pools of peritumoral cirrhotic liver from HBV- or HCV-infected individuals for *GSTP1* and *IGF2*. The random distribution of the samples resulted in a concentration of the samples displaying a high degree of DNA methylation in one of the two pools.

Quantitative Accuracy and Pool Size

To investigate further the sensitivity and specificity of our approach, we used several larger pools containing a different number of tumor samples independent of their β -catenin mutation status and etiology. Three pools were constructed containing 10 samples, three pools with 15 samples, and one pool consisting of 20 samples. Methylation patterns were analyzed for *CDKN2A*, *MLH1*, and *IGF2*. No methylation was detected in any of the pools for *MLH1* in concordance with the results obtained from the individual analysis. For *CDKN2A*, methylation was found in all pools, and comparison to the average of the individual samples showed a similar quantitative accuracy independent of the pool size (Table 5). Quantitative analysis is of even greater importance for the hyper- or hypomethylation in the DMR2 of *IGF2* because methylation is present in both normal and cancerous tissues. Again, the cancer-related changes were

detected in all pools and coincided well with the data obtained from the individual samples (Table 6). Differences were again not dependent of the pool size. From these data, we can conclude that our approach has 100% specificity and 100% sensitivity if aberrant methylation is a prevalent phenomenon in the analyzed region. The pooling of DNA without prior adjustment of the concentration would further reduce the required amount of DNA and streamline the analysis. The same DNAs as above were directly pooled together in equal quantities irrespective of their concentration after bisulfite treatment. The *IGF2* DMR2 was analyzed in these samples (Table 6), and results were compared with the individual samples and the pools in which concentration of the samples had been equalized. Differences in the nonadjusted pools were clearly increased for the smaller pools, probably attributable to some samples present at higher concentrations than others. For the larger pools ($n = 20$ and $n = 26$), this effect was less pronounced, approaching the same accuracy as the pools with equal concentrations. In summary, all genes displaying differential methylation were successfully detected, and no false-positive results were generated, demonstrating the reliability and precision of our approach.

Discussion

We have shown here a novel approach for the effective screening for differential methylation between etiolog-

Table 4. Quantitative DNA Methylation Values for a Pyrosequencing Primer Analyzing 10 CpGs in the *IGF2* DMR2 in a Pool of Human Hepatocellular Carcinomas Developed on Alcohol Consumption or without Known Etiology and Analysis of the Six Samples Comprised in the Pool Individually

Sample	CpG 6	CpG 7	CpG 8	CpG 9	CpG 10	CpG 11	CpG 12	CpG 13	CpG 14	CpG 15
Pool consisting of HCCs without β -catenin mutations from alcoholic individuals or developed without known etiology	33.7	45.1	50.4	63.7	31.9	40.4	27.7	36.7	47.3	44.9
HCC 8	21.6	27.5	30.3	42.7	17.2	22.5	17.3	23.1	27.9	28.4
HCC 9	52.5	53.8	57.5	65.8	41.9	52.7	36.9	51.8	62.8	59.3
HCC 10	43.3	59.1	54.1	65.0	30.6	48.3	34.6	49.5	59.1	59.6
HCC 11	43.9	55.3	53.4	64.7	34.4	48.2	35.4	48.8	57.5	54.4
HCC 12	11.8	27.1	48.5	70.1	27.8	29.2	21.4	29.2	31.4	34.0
HCC 13	26.0	35.7	44.5	68.6	32.5	35.5	23.8	33.2	35.1	36.0
Average of individual samples	33.2	43.1	48.1	62.8	30.7	39.4	28.2	39.3	45.6	45.3
Δ Pool/individual samples	-0.5	-2.0	-2.3	-0.8	-1.2	-1.0	+0.5	+2.6	-1.7	+0.3

Values are given in percentage, and the average values of the individual samples are compared with the values obtained in the pool.

Table 5. Quantitative DNA Methylation Values for Two Pyrosequencing Primers Analyzing Seven and Six CpGs in the *CDKN2A* Promoter in Different Pools of Human Hepatocellular Carcinomas

Pool size		Primer 1							Primer 2						
		CpG 1	CpG 2	CpG 3	CpG 4	CpG 5	CpG 6	CpG 7	CpG 8	CpG 9	CpG 10	CpG 11	CpG 12	CpG 13	
<i>n</i> = 10	Pool A	24.5	26.4	18.7	22.9	20.5	14.9	24.0	25.4	25.0	20.9	26.9	20.7	23.3	
	Average	20.9	23.6	18.5	20.2	18.9	15.6	22.5	22.9	23.6	19.9	25.9	18.3	19.7	
	Difference	-3.5	-2.8	-0.2	-2.7	-1.6	0.7	-1.5	-2.5	-1.4	-1.0	-1.0	-2.4	-3.6	
	Pool B	31.6	28.1	21.6	30.2	24.4	22.6	32.1	36.4	38.2	30.6	39.7	27.6	31.0	
	Average	25.1	24.7	20.0	24.1	21.5	19.7	26.0	30.1	31.0	25.3	35.0	24.9	27.6	
	Difference	-6.5	-3.4	-1.6	-6.0	-2.9	-3.0	-6.1	-6.2	-7.2	-5.3	-4.7	-2.7	-3.4	
	Pool C	30.3	36.5	27.2	27.8	31.2	25.4	32.2	34.0	32.9	30.2	31.8	29.1	34.1	
	Average	24.5	28.0	22.2	22.3	23.0	19.4	25.2	27.7	26.7	22.3	30.7	21.5	25.2	
	Difference	-5.8	-8.6	-5.0	-5.4	-8.2	-6.0	-7.0	-6.3	-6.2	-7.9	-1.1	-7.6	-8.9	
<i>n</i> = 15	Pool D	27.9	30.3	24.5	25.3	18.1	16.2	28.9	32.0	31.2	24.8	32.4	26.8	29.5	
	Average	24.6	26.4	23.1	23.4	22.0	20.4	25.7	28.3	29.0	24.1	30.7	22.7	24.2	
	Difference	-3.3	-3.9	-1.5	-1.9	3.9	4.2	-3.2	-3.7	-2.3	-0.7	-1.7	-4.0	-5.3	
	Pool E	33.6	36.9	21.4	33.3	33.6	24.4	35.1	36.4	35.6	22.4	38.2	29.6	33.7	
	Average	29.7	33.6	27.7	28.4	30.2	24.0	30.2	32.9	32.7	27.2	37.6	26.0	28.1	
	Difference	-3.9	-3.3	6.2	-4.9	-3.4	-0.4	-4.8	-3.5	-2.8	4.9	-0.5	-3.6	-5.6	
	Pool F	31.2	35.4	24.6	29.9	29.2	22.0	31.8	34.3	34.4	31.7	38.6	28.5	30.8	
	Average	26.5	30.8	24.2	24.8	23.8	19.9	27.1	29.7	29.9	24.6	33.7	22.7	26.0	
	Difference	-4.7	-4.6	-0.5	-5.1	-5.4	-2.1	-4.7	-4.5	-4.5	-7.1	-4.9	-5.8	-4.8	
<i>n</i> = 20	Pool G	39.1	40.9	24.5	35.8	32.3	25.2	37.7	39.9	38.6	37.1	41.5	34.3	39.4	
	Average	32.1	35.1	28.4	30.2	31.0	25.4	33.0	37.6	37.3	30.6	40.8	28.8	31.8	
	Difference	-7.0	-5.8	4.0	-5.6	-1.3	0.2	-4.7	-2.3	-1.4	-6.5	-0.7	-5.5	-7.6	

Pools were constructed from a variable number of samples (10, 15, or 20) in a blinded manner. Values are given in percentage, and the average values of the individual samples are compared with the values obtained in the pool. The results demonstrate that the accuracy of the pooled approach is independent of pool size and pyrosequencing primer.

ical homogeneous pools of clinical specimens and validated successfully this approach by the subsequent analysis of all samples individually. Three of the five genes (*CDKN2A*, *GSTP1*, and *IGF2*) displayed epigenetic plasticity in the analyzed region, which was confirmed by the individual analysis of the samples. Methylation patterns of the pooled samples reflect with high precision the average of the DNA methylation patterns of the individual samples contained in the respective pool.

Two genes (*MLH1* and *CTNNB1*) did not display any variation in the DNA methylation patterns or levels either between pools or in the individual samples. These results clearly demonstrate the ability of our approach to identify genes with variable methylation patterns with high reliability. The omission of these two genes in the individual samples would thereby have contributed to significant reduction in labor and cost of the analysis or would have permitted the inclusion of other genes.

The negative results for *CTNNB1* were anticipated because this gene is expressed in a subtype of human HCCs attributable to a genetic mutation preventing degradation of the protein and is so far not known to be regulated by epigenetic mechanisms. It was included as a negative control in this study to test for the detection of false-positive results in the pools. Hypermethylation of the CpG island spanning the transcription start of *MLH1* seems to be a rare event. One study found methylation in ~10% of human HCCs with hepatitis viral infection whereas normal livers, including noncancerous regions of livers of patients with HCC, were consistently unmethylated.³³ The negative results in our study can be explained by the comparatively low number of virus-infected HCCs in our study, phenotypic differences between the samples of Japanese and French origin, respectively.

As a critical negative regulator of cell-cycle progression, *CDKN2A* is frequently inactivated by promoter

Table 6. Differences in Percentage between the Average DNA Methylation Values for a Pyrosequencing Primer Analyzing Five CpGs in the *IGF2* DMR2 in Pooled DNA Samples Compared with the Individual Samples Contained in the Pool

Pool size	Δ Pool/individual samples									
	Concentration not adjusted					Concentration adjusted				
	CpG 2	CpG 3	CpG 4	CpG 5	CpG 6	CpG 2	CpG 3	CpG 4	CpG 5	CpG 6
<i>n</i> = 5	-9.8	-13.9	-10.7	-7.2	-10.7	5.1	4.7	0.6	3.1	3.8
<i>n</i> = 10	-4.8	4.2	3.6	1.2	7.2	-4.8	-3.4	0.8	1.9	0.3
<i>n</i> = 15	2.9	7.9	10.0	4.7	8.0	2.1	0.3	-1.2	0.1	1.1
<i>n</i> = 20	1.0	2.5	5.7	7.8	4.8	-2.8	-3.2	1.5	1.8	-1.6
<i>n</i> = 26	-0.7	0.9	4.0	6.8	2.8	3.0	-0.2	4.6	2.1	2.6

Pools consist of a variable number (5, 10, 15, 20, or 26) of human hepatocellular carcinomas. Pools are constructed with (columns to the right) or without adjustment (columns to the left) of the DNA concentration after bisulfite treatment.

methylation in a variety of human tumors. We found hypermethylation in 67% of HCCs with a significant difference toward higher methylation levels in HCCs with β -catenin mutations. In contrast to other studies³⁹ we did not find *CDKN2A* methylation in matched normal tissues infected with the hepatitis virus and adenomas, and only one of the cirrhotic livers was clearly methylated. The discrepancy is probably because of the choice of technology because most other studies used standard methylation-specific PCR, which might also amplify and detect very low levels of methylation that cannot be detected by pyrosequencing. On the contrary the quantitative methylation differences between the two groups of HCCs have to our knowledge not been previously described and would not have been detected using conventional methylation-specific PCR. *GSTP1* was found to be methylated in nearly all cancer tissues and to a lower degree in a large proportion (80%) of normal liver and matched non-cancerous liver tissue from HCC patients. The high prevalence of methylation in normal liver could be attributable to the fact that only low levels of *GSTP1* mRNA are found in adult liver (<http://symatlas.gnf.org/SymAtlas/>, accessed October 7, 2007),⁴⁰ and immunohistochemical analysis demonstrated that expression of the protein is restricted to bile duct epithelial cells whereas other cells types of the liver do not have detectable levels of the protein.^{31,41} *GSTP1* might therefore become partially methylated. A second possibility would be that the methylation could also be attributable to aging. Unfortunately, no information on this parameter was available. Analysis of the samples with a second independent primer set confirmed the high degree of methylation found in the normal liver samples, and normal breast and colon tissue that express *GSTP1* showed no methylation (<4%; J.T., unpublished data). A recent study found a similar repartition with *GSTP1* methylated in 100% of HCCs and 90.5% of matched noncancerous tissue, but also 75% of normal liver tissue.⁴² The high prevalence of DNA methylation in normal tissue makes methylation at the *GSTP1* promoter an unsuitable marker for diagnostic purposes. Nonetheless for the purpose of the validation of our pooled analysis, excellent concordance of pooled and individual samples was achieved. The observed hypomethylation in the *IGF2* DMR2 is in good concordance with previously published results studying the same region.³² However, the published results could only distinguish globally different methylation patterns in the DMR because of the use of denaturing high-performance liquid chromatography (dHPLC) as method for read-out rather than a method with quantitative single nucleotide resolution as presented here. The slight, but consistent and significant, reduction of DNA methylation levels in the DMR2 in cirrhotic liver samples compared with normal livers, which was already detected in the pooled samples, demonstrates the quantitative resolution and power of our approach. The methylation changes might be correlated to the decreased serum *IGF2* levels that have been postulated to be a sensitive and effective indicator for liver dysfunction.⁴³ However, further studies are necessary to analyze this reduction, especially with respect to differential promoter usage as well as to elucidate the two

concurrent phenomena of hyper- and hypomethylation, which are evident in cancer samples and that might in an unfortunate situation counterbalance each other. In this case no change would be detected in the analysis of pooled samples. In the present study hypomethylation was much more prevalent, thereby not confusing the analysis and detection of variable methylation patterns. However, it might be necessary for the analysis of imprinted genes to add several individual samples already in the first screening step to detect the simultaneous occurrence of hyper- and hypomethylation.

Pyrosequencing-based DNA methylation analysis relies on a candidate gene approach thus requiring a hypothesis or preliminary data to choose a limited number of genes and/or gene regions of potential interest. It aims at the identification of nonrare methylation events in a specific pathology, ie, alterations that occur in a large number of cells within a tumor and might directly contribute to—or are a nonrandom result of—tumorigenesis rather than passenger epimutations. Besides cost and effort necessary for a study, the amount of available clinical samples imposes major restrictions on the number of genes that can be analyzed. Although a growing number of genes that are inactivated by DNA methylation during carcinogenesis are being discovered, etiological, environmental, or geographic differences often require verification in a sample collection under investigation. In addition, because ~10% of all genes are postulated to be aberrantly methylated in different types or stages of tumors,⁶ a large number of genes still needs to be identified. The use of a prescreening step such as the method proposed here slightly increases time and effort for a given project. However, this is easily compensated by the concentration on differentially methylated regions between pools, and most, if not all, subsequently analyzed genes will display some difference in their methylation profile that can be used for diagnostics and/or correlated to a variety of clinical parameters. This significantly increases quality and quantity of the results of a study. Pyrosequencing of sample pools for the detection of differential DNA methylation helps to prioritize target genes. Parameters such as pool size can be adapted to capture anticipated quantitative differences between pools. Larger pools containing DNA of up to 26 samples were well suited to analyze aberrant DNA methylation present in a large proportion of samples as demonstrated in this study for *CDKN2A* or *IGF2*. In this setting, the devised approach displayed a sensitivity and specificity of 100%. However, methylation events occurring at low frequency might be missed in large pools because the quantitative resolution is not sufficient to identify the subtle changes attributable to a single or very few differentially methylated samples. The method works best if knowledge as detailed as possible is available on the samples used to classify them into clinically or pathologically homogeneous groups to answer a specific question. The composition of pools might therefore be different for distinct questions. Additional pools of identical etiology can increase the confidence in the results of the prescreen and might also detect less frequent methylation events. One important advantage of our method is

that less DNA is consumed, permitting use of the same amount of available samples to analyze methylation patterns in more genes and concentrate on the genes that actually display differential methylation. If only frequently hyper- or hypomethylated genes are of interest, the additional step of adjusting the concentration of bisulfite-treated DNAs might be omitted. The gain of rapidity and saving of DNA material, however, has to be balanced against decrease in accuracy. The demonstrated method enables rapid and accurate identification of genes functionally implicated in carcinogenesis and genes whose methylation pattern present a valuable biomarker for the early diagnosis, classification, prognosis, and therapy of human cancers.

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