Distinct Methylation Patterns of Benign and Malignant Liver Tumors Revealed by Quantitative Methylation Profiling

Ulrich Lehmann,¹ Ina Berg-Ribbe,¹ Luzie U. Wingen,² Kai Brakensiek,¹ Thomas Becker,³ Jürgen Klemptnauer,³ Brigitte Schlegelberger,² Hans Kreipe,¹ and Peer Flemming¹

Abstract Purpose: A comparative quantitative methylation profiling of hepatocellular carcinoma and the most frequent benign liver tumor, hepatocellular adenoma, was set up for the identification of tumor-specific methylation patterns.

Experimental Design: The quantitative methylation levels of nine genes (RASSF1A, cyclinD2, p16INK4a, DAP-K, APC, RIZ-1, HIN-1, GSTπ1, SOCS-1) were analyzed in hepatocellular carcinoma and adjacent normal tissue (n = 41), hepatocellular adenoma and adjacent normal tissue (n = 26), focal nodular hyperplasia (n = 10), and unrelated normal liver tissue (n = 28). Accumulated methylation data were analyzed using various statistical algorithms, including hierarchical clustering, to detect tumor-specific methylation patterns.

Results: Cluster analysis revealed that hepatocellular adenoma displays a methylation profile much more similar to that found in normal liver tissue and focal nodular hyperplasia than to that found in hepatocellular carcinoma. Many characteristic differences were not detected when using mere qualitative methylation assays. The cyclinD2 gene was identified as a new and frequent target for aberrant hypermethylation in hepatocellular carcinoma (68%). In the control group of 28 liver specimens from healthy donors, a clear correlation between age of patient and frequency and level of aberrant methylation was seen, which could not be detected in the group of hepatocellular carcinoma specimens.

Conclusions: Methylation profiling can clearly contribute to the unequivocal classification of suspicious lesions, but only if done in a quantitative manner applying cell type and gene-specific thresholds. In hepatocellular carcinoma, the altered methylation patterns accompanying malignant transformation override the age-dependent increase in gene methylation.

Inactivation of tumor suppressor genes is an important event contributing to the development of malignant tumors. In addition to the classic genetic mechanisms of deletion or inactivating point mutations, growth regulatory genes can be functionally inactivated without alteration of the primary sequence by methylation of cytosine residues in the promoter region. Depending on the gene and the cell type, such an alteration can be found in premalignant lesions and benign tumors (1). The potential of analyzing altered methylation patterns for the early and reliable detection of malignancy and as a new marker for prognosis and therapeutic monitoring has emerged over the last few years (2).

Epigenetic inactivation due to hypermethylation is well established for several genes in hepatocellular carcinoma (see ref. 3 and references therein) and is already detectable in potentially precancerous lesions of the liver, namely cirrhosis and dysplastic nodules (4), but as yet almost nothing is known about hypermethylation in hepatocellular adenoma, the most important benign epithelial tumor of the liver (5, 6). Only one study analyzed the methylation of the cell cycle regulators p16INK4a and p14ARF in hepatocellular adenoma (7), reporting a low frequency of epigenetic inactivation of the INK/ARF locus in hepatocellular adenoma.

The differentiation between benign liver adenoma and well-differentiated hepatocellular carcinoma (grade 1) is prognostically important because the latter has the capability of progression and metastatic spread (8) and resection is the only potentially curative therapeutic approach for small-sized carcinomas. A large retrospective study, however, revealed a significant risk of mortality for resection of liver adenomas (3 of 58, 5.2%; ref. 9). Therefore, the suspicious lesion has to be classified as precisely as possible before surgical resection can be considered to avoid any unnecessary risk.

For the differential diagnosis between hepatocellular adenoma and well-differentiated hepatocellular carcinoma, detection of chromosomal abnormalities using fluorescence in situ...
hybridization or bright-field in situ hybridization has been evaluated (10). Whether aberrant methylation patterns may contribute to this differential diagnosis has not yet been assessed. In addition, methylation profiling can identify new targets of inactivation in the process of neoplastic transformation and can contribute to the identification of a clonal relationship.

Therefore, we started to determine the epigenetic profile of hepatocellular adenoma compared with those of hepatocellular carcinoma and the regenerative lesion focal nodular hyperplasia. These methylation profiles may improve classification of liver tumors and elucidate the pathways affected by epigenetic inactivation in hepatocellular adenomas. Adjacent, morphologically unaltered liver tissue and biopsies from completely unrelated healthy liver specimens were also analyzed.

For the quantitative methylation profiling of hepatocellular adenoma, genes that were reported to be aberrantly hypermethylated in hepatocellular carcinoma in >50% of cases by at least two independent studies (p16INK4a, RASSF1A, APC, SOCS-1, GSTR1) were selected. In addition, four genes frequently inactivated in other epithelial malignancies but not yet analyzed in liver tumors or only reported in one publication were included in this study (RIZ-1, HIN1, cyclinD2, and DAP-Kinase).

Materials and Methods

Patient materials. Snap-frozen and formalin-fixed, paraffin-embedded liver specimens were retrieved from the tissue archive of the Institute of Pathology, Medizinische Hochschule Hannover, Germany, following the guidelines of the local Ethics Committee. In the case of fresh-frozen material, reference sections were H&E stained to confirm the histopathologic diagnosis and to evaluate the tumor cell content (>90%). Altogether, 26 hepatocellular adenomas with adjacent normal tissue, 10 focal nodular hyperplasia, 28 normal liver biopsies (control biopsies from organs selected for transplantation), 13 well-differentiated (grade 1), and 28 moderately to poorly differentiated (grades 2 and 3) hepatocellular carcinomas, partly with adjacent liver tissue, were retrieved (see Table 1).

For the morphologic classification of liver tumors, the recommendations of Ferrell et al. (11) were followed. Hepatocellular carcinoma lesions were graded according to the method of Edmondson et al. (12).

DNA extraction and bisulfite treatment. Genomic DNA was isolated from fresh-frozen or formalin-fixed paraffin-embedded biopsies by incubating four to six 10 μm sections with proteinase K followed by organic extraction according to standard procedures. For the separation of tumor and adjacent normal tissue from the same block, manual microdissection guided by an H&E-stained serial section was done. The bisulfite treatment of genomic DNA was done as described (13).

Quantitative methylation analysis. The real-time PCR-based quantification of methylation levels was done essentially as described (14). Up to 5 μL bisulfite-treated genomic DNA were amplified in a total reaction volume of 25 μL. All primer and probe sequences are listed in Table 2S (also available at http://www.mh-hannover.de/institute/pathologie/methylation.htm).

A stringent threshold was defined individually for every gene: the mean methylation level in the control group (unrelated healthy liver tissue, n = 28) plus 1.96 times the SD (95% confidence interval) was defined as the threshold for scoring a sample as “hypermethylated”. In the control group, 95.2% of all measurements were actually below this threshold.

Statistical analysis. All analyses were done using the R software package (version 2.0.0) and SPSS for Windows (version 11.5.1). The correlation analysis (age of patient versus methylation) was calculated using Spearman’s rank correlation test. The similarity analysis of paired fresh-frozen and formalin-fixed paraffin-embedded specimens was done with an agglomerative hierarchical cluster analysis using Euclidean distance and average linkage. Other distances or agglomerative measures gave very similar results (data not shown). Cluster analyses of methylation patterns were done in the same way. Methylation levels in the different sample groups (hepatocellular adenoma, liver tissue adjacent to hepatocellular adenoma, hepatocellular carcinoma, liver tissue adjacent to hepatocellular carcinoma, focal nodular hyperplasia, unrelated normal liver tissue) were compared pair wise using the Wilcoxon rank sum test. Methylation frequencies in two groups were compared using Pearson’s χ² test. For prediction of classifiers that best characterize the different classes, the prediction analysis for microarrays (PAM) algorithm (15) was applied to different subsets of the continuous data (PAM library for R).

Results

Selection of genes and establishment of quantitative methylation assay. A systematic analysis of the literature was done to identify genes with a high frequency of hypermethylation in hepatocellular carcinoma. Those genes with a clear methylation in >50% of cases found by two independent groups (and showing also high methylation rates in a prescreen in our laboratory) were selected: p16INK4a, RASSF1A, APC, SOCS-1, and GSTR1. For all five genes, aberrant hypermethylation is also described in a wide variety of epithelial malignancies (see ref. 16 and references therein). In addition, we analyzed the following genes: RIZ-1, HIN1, cyclinD2, and DAP-Kinase. All four genes are described to be epigenetically inactivated in several epithelial malignancies (e.g., breast, lung, stomach; refs. 17–20) but, thus far, are not analyzed in liver tumors (cyclinD2, HIN1) or only in one study (RIZ-1, DAP-Kinase; refs. 18, 21).

For five target genes (GSTR1, APC, RIZ-1, HIN1, and SOCS-1), new real-time PCR-based methylation assays were developed and validated according to the criteria described elsewhere in detail (14). For the selection of primer and probe sequences, all published and unpublished data were taken into account for the identification of the cytosine residues that are actually methylated in primary tumor biopsies and whose methylation is linked to transcriptional silencing. Measuring target and reference genes over a broad range of DNA concentrations and a wide spectrum of methylation levels, a

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Table 1. Liver specimens analyzed in this study

<table>
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<tr>
<th></th>
<th>Normal liver</th>
<th>HCA</th>
<th>FNH</th>
<th>Total</th>
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<td>26</td>
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</tr>
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<td>—</td>
<td>—</td>
<td>13/28</td>
<td>7/10</td>
<td>6/18</td>
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Abbreviations: HCA, hepatocellular adenoma; FNH, focal nodular hyperplasia; HCC, hepatocellular carcinoma.

*In three cases, sex of liver donor was not known; in one case, age was unknown.

4 U. Lehmann et al., unpublished results.
constant ratio of reference and target gene ($\Delta C_T = C_{\text{Target}} - C_{\text{Reference}}$) and a very good linear correlation between methylation level and measured $\Delta C_T$ could be shown for all five newly established assays (Fig. 1).

Suitability of formalin-fixed, paraffin-embedded biopsies for methylation analysis. From the series of 26 pairs of liver cell adenoma and adjacent normal tissue, 24 sample pairs were formalin-fixed and paraffin-embedded biopsies from 1987 to 2003. All of them were suitable for molecular analysis.

For seven samples (four hepatocellular adenoma and three adjacent normal tissue specimens), snap-frozen and formalin-fixed paraffin-embedded biopsies were available in parallel. Only one pair of specimens showed moderate quantitative differences in two of nine methylation assays. All other measurements showed identical or nearly identical methylation levels (data not shown).

Age-related hypermethylation in normal liver tissue. The analysis of 28 biopsies from normal liver tissue (control biopsies from organs selected for transplantation) supports the observation that gene methylation increases with age (22) because the only three tissue samples without any signs of methylation are from patients younger than 4 years old. A statistical analysis revealed a strong correlation between patient age and the occurrence of gene methylation in normal liver specimens (Spearman’s rank correlation: 0.67, $P = 0.0002$).

Advantages of quantitative methylation detection. The widely used mere qualitative detection of DNA methylation is in many cases not able to differentiate normal tissue or benign lesions from malignant tumors. The RASSF1A gene, for instance, shows a weak but clearly detectable methylation signal in normal liver tissue and focal nodular hyperplasia specimens in 57% and 70% of cases, respectively, using sensitive qualitative assay conditions (hepatocellular carcinoma: 98%, see Fig. 2). With a stringent threshold only 3.5% of the normal samples (23, 24), the hepatocellular carcinoma specimens harbor in 16 of 41 cases (39%) extensive SOCS-1 hypermethylation.

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Identification of cyclinD2 as a novel target for hypermethylation in hepatocellular carcinoma. The cyclinD2 gene shows a very high frequency of moderate to strong hypermethylation in hepatocellular carcinoma (in 68% of cases, with the above-defined level as a threshold). Epigenetic inactivation of this gene has been reported as yet only for breast cancer (14,17), prostate, lung, pancreatic, and gastric cancer (25–28). The frequency of methylation found for the cyclinD2 gene in hepatocellular carcinoma is higher than in all other malignancies thus far described for this gene.

Age-independent hypermethylation in hepatocellular carcinoma. Because we found a strong correlation between age and hypermethylation events in normal liver tissue, the high incidence of aberrant methylation in hepatocellular carcinoma specimens compared with the hepatocellular adenoma and focal nodular hyperplasia specimens could be, at least in part, due to the older age of the average hepatocellular carcinoma patient (see Table 1). To exclude this potentially confounding factor, we compared separately hepatocellular carcinoma specimens from patients 40 years old or younger versus specimens from patients older than 40 years using different statistical methods including the cluster algorithms mentioned below. No significant differences between these two groups of hepatocellular carcinoma patients could be found (data not shown). Also, no correlation between age and frequency of hypermethylation in hepatocellular carcinoma specimens could be detected (Spearman's rank correlation: 0.16, P = 0.31).

Distinct methylation patterns in adjacent nonneoplastic liver tissue. In liver tissue adjacent to hepatocellular adenoma, the APC gene is methylated more frequently and to a higher extent than in the biopsies from unrelated normal liver specimens. The same holds true both for the cyclinD2 gene and the RASSF1A gene in the case of nonneoplastic liver tissue adjacent to hepatocellular carcinoma (P = 0.04 and P = 0.004, respectively, Wilcoxon rank sum test). Therefore, the liver tissue adjacent to a tumor clearly harbors alterations in the DNA methylation patterns that are related to aberrant hypermethylation in the tumor tissue.

Cluster analysis of methylation patterns. To determine whether a molecular classification of liver lesions could be achieved by the identified methylation patterns, agglomerative cluster analyses were conducted using different distance and clustering measure algorithms. These analyses revealed no clear differences between hepatocellular adenoma, the adjacent liver tissue, unrelated normal liver tissue, and focal nodular hyperplasia, whereas the great majority of hepatocellular carcinoma specimens clustered separately (Fig. 4). To identify discriminating methylation patterns not detected by cluster analysis, several classification techniques were applied.

The highest number of discriminating genes was found, as expected, between hepatocellular carcinoma and unrelated normal liver tissue (RASSF1A, cyclinD2, p16INK4a, GSTπ1, APC, RIZ1, SOCS-1; Wilcoxon test, P < 0.008). A very similar
set of genes discriminates between hepatocellular adenoma and hepatocellular carcinoma (RASSF1A, cyclinD2, GSTπ1, APC, RIZ1, SOCS-1; Wilcoxon test, P < 0.04).

For prediction of those methylation events that best characterize the different entities analyzed in this study, PAM was used (see Materials and Methods). This analysis identified a set of only three genes (RASSF1A, cyclinD2, APC) with a very promising prediction ratio for the discrimination between hepatocellular adenoma and hepatocellular carcinoma: the overall error rate was 0.163.

Fig. 3. Frequency of multiple methylation events in normal liver tissue and different epithelial liver tumors. Multiple methylation is defined as concomitant hypermethylation of three or more genes in one sample. Below each entity, the average number of methylated genes is indicated. Adj, adjacent nonneoplastic liver tissue. A, any methylation signal was taken into account, regardless of the quantitative methylation level. B, frequency of multiple methylation if only signals above a threshold of mean[control] + 1.96 × SD were scored.

Discussion

This study represents the first comprehensive comparison of the methylation profiles of benign and malignant epithelial liver tumors. For this purpose, a large series of hepatocellular adenoma, the most important benign liver tumor, was compared with well and poorly differentiated hepatocellular carcinoma, the regenerative lesion focal nodular hyperplasia, and unrelated normal liver tissue. Also, morphologically inconspicuous liver tissue adjacent to the selected tumors was analyzed.

To determine whether the overall methylation patterns of all nine genes analyzed in this study display specific differences, cluster analyses were done. These algorithms have only recently been used for the analysis of complex sets of methylation data (29, 30).

This cluster analysis revealed that hepatocellular adenoma display a methylation profile much more similar to normal liver tissue and focal nodular hyperplasia than to hepatocellular carcinoma (Fig. 4). This is further evidence on the epigenetic level of altered DNA methylation patterns for the concept that hepatocellular adenoma is not a direct precursor of hepatocellular carcinoma. Alternatively, these results imply that transition from hepatocellular adenoma to hepatocellular carcinoma involves significant epigenetic evolution. Conventional statistical analysis (Wilcoxon rank sum test) revealed that the highest number of statistically significant differences in methylation were found for the comparison of hepatocellular carcinoma with normal liver tissue (RASSF1A, cyclinD2, p16INK4a, GSTπ1, APC, RIZ1, SOCS-1) followed by the number of genes discriminating between hepatocellular adenoma and hepatocellular carcinoma (RASSF1A, cyclinD2, GSTπ1, APC, RIZ1, SOCS-1).

The malignant transformation of a bona fide hepatocellular adenoma in a premenopausal woman (the main risk group) is still not resolved in the literature. A careful and comprehensive meta-analysis (31) reports only one unequivocal case of a postmenopausal woman and another case arising in a multinodular adenoma. Hepatocellular carcinoma in a young woman after occurrence of an adenoma in early childhood has also been reported (32). Regarding the extreme rarity of these cases, the authors themselves raise the question of coincidence rather than of clonal relationship.

The three genes identified by PAM as having the greatest discriminatory power (RASSF1A, cyclinD2, APC) affect three different cellular functions: cell division (RASSF1A; ref. 33), cell cycle (cyclinD2; ref. 34), and signal transduction (APC; ref. 35). Therefore, the synergistic effect of the gene products could drive selection of these three complementary defects.

The frequency of cyclinD2 hypermethylation is among the highest reported for any gene in hepatocellular carcinoma thus far (68% of cases). This frequency is also higher than in any other malignancy for which cyclinD2 gene hypermethylation is described (17, 19, 25–28). Therefore, the current profiling approach identified a new frequently hypermethylated gene in hepatocellular carcinoma, which might be a promising candidate as a marker for differential diagnosis as well as a new potential serum marker for hepatocellular carcinoma (4, 36, 37).

The results illustrated in Figs. 2 and 3 show that the qualitative detection of methylation can mask huge differences.
in the extent of methylation and is very often not capable of discriminating between different entities. Only the application of defined and stringent thresholds revealed statistically highly significant differences in the methylation frequencies for genes like cyclinD2 or RASSF1A in liver cell adenoma, normal liver tissue, or regenerative lesions (focal nodular hyperplasia) compared with hepatocellular carcinoma (Fig. 3). Therefore, a quantitative real-time PCR-based assay was used in this study. Also, the strictly qualitative detection of methylation by the widely used conventional methylation-specific PCR is hampered by several additional shortcomings: (a) The reaction efficiencies and, thereby, the detection sensitivity of qualitative methylation-specific PCR is not known exactly. (b) The frequency of hypermethylation might be overestimated using conventional methylation-specific PCR (38, 39). (c) The methylation assay using real-time PCR is more stringent than the conventional methylation-specific PCR, because, in addition to the two primers specific for methylated DNA, the probe also has to anneal correctly with the target sequence. (d) The results of nonstandardized qualitative assays from different laboratories are difficult to compare. Concerning the threshold of detection for scoring a sample as methylated, a consensus is not yet reached in the literature. The values vary between 0.2% and 20% (40–46); only Fackler et al. (46) apply, in a recently published study of breast cancer, gene-specific thresholds for the RASSF1A gene. Due to the small sample size (only for 21 of 41 hepatocellular carcinoma specimens adjacent tissue was available), no clear correlation between methylation levels in normal-appearing adjacent tissue and carcinoma cells could be detected (only a statistical trend).

In future studies, it would be desirable to systematically analyze liver specimens from different geographic areas, as initiated by Shen et al. (49), to elucidate the influence of different etiologic factors and varying genetic background on the development of aberrant methylation patterns.

Acknowledgments

We thank Kathleen Metzig and Britta Hasemeier for their skilful technical support and Gillian Tricke for improving the English of this article.

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