Epigenetic defects of hepatocellular carcinoma are already found in non-neoplastic liver cells from patients with hereditary haemochromatosis

Ulrich Lehmann1,*, Luzie U. Wingen2, Kai Brakensiek1, Heiner Wedemeyer3, Thomas Becker4, Albert Heim5, Kathleen Metzig1, Britta Hasemeier1, Hans Kreipe5 and Peer Flemming1

1Institute of Pathology, Medizinische Hochschule Hannover and 2Department of Disease and Stress Biology, John Innes Centre, Colney Lane, Norwich NRE 7UH, UK, 3Department of Gastroenterology, Hepatology, and Endocrinology, 4Department of Visceral and Transplantation Surgery and 5Institute of Virology, Medizinische Hochschule Hannover, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany

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Gene silencing through aberrant CpG island methylation is a frequent epigenetic defect in hepatocellular carcinoma (HCC). However, nothing is known as yet whether aberrant hypermethylation occurs already in non-neoplastic liver cells from patients with hereditary haemochromatosis who have a clearly elevated risk for developing HCC. Therefore, quantitative real-time PCR-based methylation analysis of six genes frequently hypermethylated in HCC (RASSF1A, cyclinD2, p16INK4a, GSTp1, SOCS-1, APC) was performed for liver biopsies from patients with hereditary haemochromatosis. For genotyping of the HFE gene restriction enzyme analysis and Pyrosequencing were used. Transcriptional repression of hypermethylated genes was assessed using real-time RT–PCR. Eighty-four percent of all samples with severe hepatic iron overload and a mutated HFE gene (but without HCC) had at least one gene hypermethylated. All six genes tested were affected by aberrant hypermethylation, albeit to a different extent: RASSF1A 55%, cyclinD2 45%, p16INK4a 32%, GSTp1 10%, SOCS-1 6%, APC 8%. Concomitant transcriptional down-regulation was shown for RASSF1A, cyclinD2, GSTp1 and SOCS-1. Biopsies from haemochromatosis patients showed significantly more aberrant hypermethylation than normal liver tissue or benign liver tumours (P < 0.001) and also to a higher degree. This effect is independent of patient age, cirrhosis or hepatitis infection. This is the first report demonstrating that longstanding severe iron overload is frequently associated with epigenetic defects characteristic of HCC, which reflects the increased risk of these lesions to progress to HCC. Thus, changes in DNA methylation patterns are an early event preceding morphological alterations of malignant transformation and represent promising targets for early detection.

INTRODUCTION

Inactivation of tumour suppressor genes is an important event contributing to the development of malignant tumours. In addition to the classical 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. These alterations can already be found in pre-malignant lesions and non-invasive carcinomas indicating that DNA methylation changes are an early event in the process of malignant transformation (1,2). The potential of analysing altered methylation patterns for the early detection of malignancy and as a new marker for prognosis and therapeutic monitoring has emerged over the last few years (3). Epigenetic inactivation due to hypermethylation is well established for several genes in hepatocellular carcinoma

*To whom correspondence should be addressed at: Institute of Pathology, Medizinische Hochschule Hannover, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany. Tel: +49 5115324501; Fax: +49 5115325799; Email: lehmann.ulrich@mh-hannover.de

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(HCC) (4,5) and is already detectable in potentially pre-cancerous lesions of the liver (6,7). However, the extent and frequency of aberrant methylation in these pre-cancerous lesions is still under discussion (8). Clearly distinct from these patterns, regenerative lesions such as focal nodular hyperplasia (FNH) and benign liver tumours such as hepatocellular adenoma (HCA) display methylation profiles which are much more similar to that found in normal liver (9).

Another condition associated with an increased risk for progression to HCC is chronic severe iron overload, as seen in hereditary haemochromatosis (10). Affected patients have a 20- to 200-fold increased risk to develop HCC (11–13). The reports of HCC in haemochromatosis patients in the absence of cirrhosis are a strong argument for an independent carcinogenic role of iron in the liver (14–16). However, as yet nothing is known about epigenetic alterations in haemochromatosis, which might support their pre-cancerous nature and might give additional clues about pathways and mechanisms involved. In addition, these alterations represent promising targets for early detection. Therefore, we started to determine the epigenetic profile of liver biopsies from haemochromatosis patients displaying severe hepatic iron overload in the presence or absence of accompanying HCC and/or cirrhosis.

For the quantitative methylation profiling of haemochromatosis biopsies, genes frequently hypermethylated to a high level in HCC were selected: p16INK4a, RASSF1A, APC, SOCS-1, GSTπ1, cyclinD2 [see Lehmann et al. (9) and references therein]. Functional consequences of hypermethylation were assessed by real-time RT–PCR for exact transcript quantification.

RESULTS

Patient population

For this study, only biopsies with all morphological characteristics of severe iron overload suspicious for hereditary haemochromatosis (17,18) were retrieved from the archive (hepaticcellular but not reticuloendothelial iron with histological iron score of 4+). Also, clinical data indicative of haemochromatosis [elevated serum ferritin and transferrin saturation (19)] and firmly excluding additional causes of chronic liver disease were a prerequisite. Altogether, 77 specimens with iron overload were retrieved (group I in Fig. 1). In addition, 10 cases with severe neonatal siderosis were selected (subgroup XII in Fig. 1). All samples were analysed for the two most frequent mutations associated with haemochromatosis [HFE C282Y and HFE H63D (20)] using conventional restriction fragment polymorphism (RFLP) analysis after PCR amplification and Pyrosequencing™. For 161/174 (92.5%) assays, both methods gave concordant results. The remaining cases were re-analysed using both methods, yielding a concordance of 100%. In all discrepant cases, Pyrosequencing™ turned out to be more reliable than RFLP analysis after PCR amplification. The specimens are divided into samples from patients without HCC (n = 60, subgroup II in Fig. 1) and samples from patients with HCC (n = 17, subgroup III in Fig. 1). Corresponding tumour tissue was available in 10 of these 17 cases (subgroup VI in Fig. 1). In the remaining cases, tumour tissue was not available due to extended necrosis or destruction by chemical embolization (subgroup VII in Fig. 1). The cases without HCC are divided into samples with HFE mutation (subgroup V in Fig. 1) and samples with HFE wild-type sequence (subgroup IV in Fig. 1). Special attention was paid to the selection of cases with homozygous HFE C282Y mutation and severe iron overload but without cirrhosis and only mild fibrosis (fibrosis grade F0–F2, subgroup XI in Fig. 1). All cases in this subgroup XI are also hepatitis-negative (see Supplementary Material for details).

Selection of genes for methylation analysis

Since this study addressed the question of whether epigenetic aberrations, which have been described to be characteristic of HCC, can already be found in the non-malignant, but pre-cancerous situation of chronic iron overload, a set of genes was selected which showed strong and frequent hypermethylation in HCC specimens. Those genes with a clear methylation in >50% of cases found by two independent groups (and confirmed in our laboratory) were selected: RASSF1A, p16INK4a, APC, SOCS-1 and GSTπ1. In addition, we included the cyclinD2 gene in our panel, because it was identified by our group as frequently methylated in HCC (9). For all six target genes, real-time PCR-based methylation assays were developed and evaluated as described in detail in Lehmann et al. (9).

Frequent aberrant hypermethylation in liver cells from patients with hereditary haemochromatosis

In this study, hereditary haemochromatosis is defined as follows: histologically confirmed parenchymal hepatic iron overload, homozygous HFE C282Y mutation, elevated serum ferritin and transferrin saturation and absence of other chronic liver disease (18,19,21). Of the 22 samples fulfilling this definition and without HCC (subgroup IX, Fig. 1), 82% had at least one hypermethylated gene (for individual methylation data see Supplementary Material). Especially, RASSF1A (55%) and cyclinD2 (45%) gene methylation are frequent events. The p16INK4a gene also shows frequent hypermethylation (32%) albeit to a low degree in all affected samples. The other genes under investigation are less frequently methylated: GSTπ1 10%, APC 8%, SOCS-1 6% (Table 1).

In contrast to these results, 10 samples from patients with neonatal siderosis were almost completely devoid of any hypermethylation. Only three samples showed a weak methylation of the p16INK4a gene. All other samples were negative for all genes tested (see Supplementary Material).

Since several genes already showed a considerable level of methylation in normal liver cells, a stringent threshold was applied for scoring a sample as ‘hypermethylated’ (see Materials and Methods for details), excluding constitutive methylation from further analysis.

Excluding age, cirrhosis and chronic hepatitis as potentially confounding factors

Several groups including our own (9,22,23) have described an accumulation of aberrant methylation during life span.
Therefore, different groups were age-matched as far as possible (median/mean): control 47/44.7 years, HFE mutated 53.5/51.8 years, iron overload with accompanying HCC 61/60.8 years. In addition, a possible correlation between hypermethylation and patient age at time of sampling was analysed for all different sample groups for all genes, but no correlation between frequency and extent of methylation could be found (data not shown).

From all cases without cirrhosis (Ishak score F0–F2), already 76.5% (13/17) showed aberrant methylation of at least one gene tested (see Supplementary Material). In addition, the methylation patterns of samples without cirrhosis and a homozygous HFE C282Y mutation (subgroup XI in Fig. 1) did not show any statistically significant differences from samples with a homozygous HFE C282Y mutation and severe fibrosis or cirrhosis (subgroup X in Fig. 1, $P = 0.77$, data not shown). These data show that the occurrence of aberrant hypermethylation is independent of cirrhosis in the patient population under study.

Whether chronic hepatitis B or C infection is associated with aberrant hypermethylation is still under discussion and obviously a gene-specific phenomenon. Contradictory results have been reported in the literature (7,8). This might also be due to geographical as well as methodological differences. But the presence of aberrant hypermethylation in the liver biopsies from patients with hereditary haemochromatosis was independent of the coexistence of a hepatitis infection (see Supplementary Material).

Figure 1. Overview of all liver specimens analysed in this study. Throughout the text, the numbering of the subgroups used in this figure is cited in addition to the description of the subgroup. The important subgroup of specimens harbouring a homozygous HFE mutation (C282Y) but without cirrhosis (subgroup XI) is shaded in light grey. These samples are also hepatitis negative (see Supplementary Material). The quantitative methylation data for the control group (biopsies from organs selected and found to be suitable for organ transplantation) and the benign liver tumours hepatocellular adenoma and focal nodular hyperplasia were available from the previous study (9).
Comparison with normal liver and benign liver tumours

Comparing hypermethylation in haemochromatosis biopsies (subgroup IX in Fig. 1) with normal liver tissue (subgroup XIII in Fig. 1) revealed statistically highly significant differences concerning the distribution of multiple methylation events. The RASSF1A gene, the cyclinD2 gene and the SOCS-1 gene were found to be much more frequently methylated and to a higher level in haemochromatosis biopsies when compared with normal healthy liver tissue (RASSF1A, P < 0.001; cyclinD2, P = 0.001; SOCS-1, P = 0.005). Also, biopsies from haemochromatosis patients quite frequently harbour simultaneous hypermethylation of several genes (compare Fig. 2 panel A with panel D, P < 0.001). Furthermore, in comparison to the benign liver tumour HCA (subgroup XIV in Fig. 1) or the regenerative lesion FNH (subgroup XV in Fig. 1), the specimens from haemochromatosis patients display a similar increase in the frequency and level of hypermethylation as well as the occurrence of multiple hypermethylation [methylation profiles of HCA and FNH were available from a previous study (9)]. Very similar statistical differences were found when only the haemochromatosis biopsies without cirrhosis (subgroup XI in Fig. 1) were compared with normal liver tissue, HCA or FNH specimens (see Supplementary Material, Table S2 for all P-values and Fig. 2 for distribution of multiple methylation).

Comparison with HCC and liver tissue adjacent to HCC

In general, all haemochromatosis samples showed less frequent aberrant hypermethylation in HCC (tumour samples from subgroup VI in Fig. 1) and also in comparison to the cirrhotic liver tissue adjacent to the carcinoma (subgroup VII in Fig. 1). The distribution of multiple methylation shows distinct differences (see Supplementary Material, Table S2 for all P-values) and also, when analysed individually, five out of six genes show significantly less methylation in haemochromatosis patients in comparison to samples from HCC patients (χ² test HCC versus haemochromatosis: RASSF1A P = 0.005, cyclinD2 P = 0.014, GSTp11 P = 0.001, APC P < 0.001, SOCS-1 P = 0.002, p16INK4a P = 0.153). All distribution patterns for multiple methylation events are displayed in Figure 2, and the corresponding P-values are listed in Supplementary Material, Table S2.

Comparison of the level of hypermethylation for all six genes in the 10 sample pairs with adjacent tissue and HCC (subgroup VI in Fig. 1) revealed that in 49/60 measurements the HCC shows a higher level of hypermethylation. In eight cases, similar levels were measured and in only three cases, the methylation level in the HCC is lower than in the surrounding tissue (see Supplementary Material for all individual measurements).

Assessment of functional consequences of hypermethylation

Since promoter hypermethylation is tightly linked to transcriptional silencing, the mRNA levels were measured for those genes displaying high-level methylation in several samples. For this purpose, quantitative real-time RT–PCR methodology was employed. Only for a subset of specimens, which were mostly core needle biopsies, could a sufficient amount of RNA suitable for molecular analyses be isolated (see Materials and Methods for quality criteria). As can be seen in Fig. 3, all specimens with high-level methylation displayed a strong reduction in mRNA expression of the respective gene. The majority of samples showed a good correlation between level of aberrant hypermethylation and mRNA expression level (r in the range of 0.66–0.91, Fig. 3). Not a single sample with high methylation level showed strong expression. However, for all four genes tested, a subset of samples (marked by light grey triangles in Fig. 3) shows a reduction in mRNA level of the respective gene without concomitant hypermethylation, indicating that additional mechanisms for transcriptional silencing do exist for these genes.

**DISCUSSION**

To the best of our knowledge, this is the first study addressing the question of whether non-neoplastic liver cells from patients with chronic severe (familial) iron overload harbour already epigenetic aberrations characteristic of HCC. These lesions are associated with an increased risk for the development of HCC, thereby representing a pre-cancerous condition (10). HCC may develop in patients with haemochromatosis independent of cirrhosis, since several studies reported HCC in haemochromatosis patients in the absence of cirrhosis (14–16).
Liver cells damaged by chronic severe iron overload show several epigenetic abnormalities found in HCC but not in benign liver tumours (HCA) or regenerative lesions (FNH). The identification of these epigenetic alterations in liver cells with severe iron overload but only minimal fibrosis (subgroup XI in Fig. 1) supports the concept that iron is directly involved in the development of HCC independent of other known risk factors (24). In line with this hypothesis, Furutani et al. (25) could show very recently in an animal model that iron overload contributes to HCC induction in conjunction with a persistent hepatitis C infection.

In addition, these data confirm the notion that epigenetic alterations, in this case aberrant hypermethylation, can precede the development of cirrhosis and represent a very early step in malignant transformation (2,5).

In the previous study, we could show that hypermethylation of the SOCS-1 gene is associated with an increased activity of the JAK/STAT signalling pathway in human tumour cells (26). In conjunction with inactivation of the pro-apoptotic RASSF1A gene (27) and interference with detoxification [inactivation of GSTπ1 (28)], this may lead to hyperproliferation of liver cells in the presence of genotoxic stress induced by long-standing iron overload. This will increase the likelihood of accumulation of mutations in the affected liver cells, thereby contributing to the development of HCC.

The liver tissue with severe iron overload adjacent to HCC harbours statistically significantly more aberrant methylation events than the biopsies from patients without HCC (see Table 1 and Supplementary Material, Table S2, and Fig. 2), indicating that these samples might represent a more advanced stage in the development of HCC. In 49/60 (82%) measurements, the methylation level for a given gene is higher in the HCC compared to the surrounding tissue (subgroup VI in Fig. 1), further confirming the progressive nature of this epigenetic defect.

The almost complete absence of any aberrant hypermethylation in the liver samples from patients with severe neonatal siderosis (subgroup XII in Fig. 1, see Supplementary Material) reflects most probably the requirement for a longstanding chronic exposure to iron overload in order to accumulate epigenetic defects in the affected liver cells.

Loss of expression in the absence of hypermethylation (as shown in Fig. 3, light grey triangles) could be due to genomic alterations (deletion or mutation) or regulatory mechanisms. This has to be addressed in future studies. Also, the influence of promoter hypermethylation on mRNA stability has to be analysed in future studies because reduced mRNA stability cannot be excluded formally as an additional factor contributing to reduced mRNA levels in primary patient samples.

Frequencies of aberrant hypermethylation reported in this study might differ for one or the other gene from reports in the literature due to technical and epidemiological reasons: in contrast to the widely used conventional Methylation Specific PCR (MSP), quantitative methylation assays with stringent thresholds were employed (see Materials and Methods for details). Also, geographical differences for the occurrence of hypermethylation in liver tumours are described in the literature (6).
The clear differences in frequency and extent of aberrant methylation in liver cells from patients with hereditary haemochromatosis compared to normal liver and benign liver tumours were only detectable by employing quantitative techniques for the detection of methylation and the application of stringent thresholds. This demonstrates again the requirement for quantitative methylation analysis (9).

In conclusion, we could show for the first time that chronic exposure of liver cells to iron overload is associated with epigenetic alterations characteristic of HCC, reflecting the increased risk of these lesions to progress to HCC. These epigenetic defects occur very early in hepatocarcinogenesis, independent of the presence of cirrhosis, hepatitis infection or patient age and represent promising targets for early detection and prognosis.

In future prospective studies, the influence of successful therapy of haemochromatosis on methylation patterns should be evaluated in order to assess the reversibility of this epigenetic alteration in vivo and to assess at which stage these changes represent an irreversible damage of the genome.

**MATERIALS AND METHODS**

**Patient materials and selection of samples**

For this study, only biopsies with all morphological characteristics of iron overload suspicious for hereditary haemochromatosis (18,21) were retrieved from the archives of the Institute of Pathology, Medizinische Hochschule Hannover, Hannover, following the guidelines of the local Ethics Committee (severe diffuse hepatocellular but not reticuloendothelial iron with histological iron score of 4+). Also, clinical data indicative of haemochromatosis [elevated serum ferritin and transferrin saturation (19)] and firmly excluding additional causes of chronic liver disease were a pre-requisite.

Altogether, 62 specimens with iron overload but without HCC and 17 specimens with accompanying HCC were retrieved. In addition, 10 cases with severe neonatal siderosis were selected.

Fibrosis was graded according to Ishak et al. (29). Fresh tissue samples for quantitative assessment of hepatic iron content were not available for this retrospective study. However, previous studies could demonstrate a good correlation between biochemical iron determination and the histological evaluation, especially when only cases with severe diffuse iron overload were considered, as in this study (17,30).

Methylation data for 26 HCA with adjacent tissue, 10 FNH and 28 unrelated normal liver specimens from organs selected and found to be suitable for transplantation were available from a previous study (9).

**DNA extraction and bisulphite 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 (31). For the separation of tumour and adjacent normal tissue, manual microdissection guided by an H&E-stained serial section was performed. The bisulphite treatment of genomic DNA was performed as described previously (32).

Mutation analysis of HFE gene
The presence of HFE C282Y and HFE H63D mutations was detected by RFLP analysis as described (33). In addition, the mutational status was determined using Pyrosequencing technology, which allows the precise quantitative analysis of polymorphic sites (34). All primers used are listed in Supplementary Material, Table S1.

Quantitative methylation analysis
The real-time PCR-based quantification of methylation levels was performed essentially as described (35). Up to 5 μl of bisulphite-treated genomic DNA were amplified in a total reaction volume of 25 μl. All primer and probe sequences are listed in Supplementary Material, Table S1. The methylation-independent amplification of β-actin and col2I is used for normalization of DNA input in all methylation assays.

The mean methylation level in a control group (unrelated healthy liver tissue, n = 28) plus 1.96 times the standard deviation was defined as the threshold for scoring a sample as ‘hypermethylated’ [for details see reference (9)].

Quantitative real-time RT–PCR
For the determination of mRNA expression levels, quantitative real-time RT–PCR was employed. RNA extraction from formalin-fixed, paraffin-embedded liver biopsies, cDNA synthesis and real-time PCR were performed essentially as described (36,37). Only those specimens were analysed using real-time RT–PCR for which the cDNA equivalent of 50 ng RNA produced a reproducible signal after a maximum of 33 PCR cycles (C<sub>T</sub>-value ≤33) for the housekeeping gene message. Two independent cDNA preparations were tested for each specimen.

All primers and probes used are listed in Supplementary Material, Table S1.

Statistical analysis
All analyses were performed using SPSS for Windows (version 13.0.0). For comparison of frequency and extent of methylation in different groups, χ<sup>2</sup> and Mann–Whitney U tests were used (two-sided, exact). P-values of less than 0.05 were considered statistically significant.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG Online.

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Conflict of Interest statement. All authors declare that no competing interests exist.

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