Neumann, O., Kesselmeier, M., Geffers, R., Pellegrino, R., Radlwimmer, B., Hoffmann, K., Ehemann, V., Schemmer, P., Schirmacher, P., Lorenzo Bermejo, J., Longerich, T. Methylome analysis and integrative profiling of human HCCs identify novel protumorigenic factors (2012) Hepatology, 56 (5), pp. 1817-1827.

Methylome analysis and integrative profiling of human HCCs identify novel protumorigenic factors.

Olaf Neumann[1], Miriam Kesselmeier[2], Robert Geffers[3], Rossella Pellegrino[1], Bernhard Radlwimmer[4], Katrin Hoffmann[5], Volker Ehemann[1], Peter Schemmer[5], Peter Schirmacher[1], Justo Lorenzo Bermejo[2], and Thomas Longerich[1]

[1]Institute of Pathology, University Hospital Heidelberg, Heidelberg, Germany
[2]Institute of Medical Biometry and Informatics, University Hospital Heidelberg, Heidelberg, Germany
[3]Genome Analytics, Helmholtz Centre for Infection Research, Braunschweig, Germany
[4]Division of Molecular Genetics, German Cancer Research Centre, Heidelberg, Germany
[5]Department of General Surgery, University Hospital Heidelberg, Germany

Keywords: methylation, profiling, hepatocellular carcinoma, tumor suppressor gene

Corresponding author
Thomas Longerich, MD
Institute of Pathology
University Hospital Heidelberg
Im Neuenheimer Feld 224
69120 Heidelberg
Tel: +49-6221-564173
Fax: +49-6221-565251
Email: thomas.longerich@med.uni-heidelberg.de

Abbreviations

5-aza-dC	5-aza-2'-deoxycytidine
aCGH	array-based comparative genomic hybridization
ATM	ataxia telangiectasia mutated
CDKN2A	cyclin-dependent kinase inhibitor 2A (p16)
CHEK2	CHK2 checkpoint homolog
Chr.	Chromosome
CI	confidence interval
CTNNA3	catenin (cadherin-associated protein), alpha
CTNNB1	catenin (cadherin-associated protein), beta
DAB2IP	DAB2 interacting protein
DNA	deoxyribonucleic acid
DNM3	dynamin 3
FDR	false discovery rate
FLT4	fms-related tyrosine kinase 4
FOXE3	forkhead box E3
GPR25	G protein-coupled receptor 25
GRASP	GRP1-associated scaffold protein
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCC	Hepatocellular carcinoma
HOXA9	homeobox A9

HOXD8	homeobox D8
HTR7	5-hydroxytryptamine receptor 7
IGFALS	insulin-like growth factor binding protein, acid labile subunit
IGF2-AS	insulin-like growth factor 2 antisense (non-protein coding)
KEGG	Kyoto Encyclopedia of Genes and Genomes
KRT73	keratin 73
LOH	loss of heterozygosity
LOI	loss of Imprinting
NETO2	neuropilin (NRP) and tolloid (TLL)-like 2
NID2	nidogen 2 (osteonidogen)
NKX6-2	NK6 homeobox 2
PARP	Poly(ADP-ribose)-Polymerase
PcG	Polycomb group protein
PCR	Polycomb Repressive Complex
PER	Period homolog family
PER3	Period homolog 3
POU4F1	POU class 4 homeobox 1
RNA	ribonucleic acid
PROZ	protein Z
RUNX3	runt-related transcription factor 3
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
siNS	nonsense siRNA
siRNA	short interfering RNA
SOCS2	suppressor of cytokine signaling 2
TBX4	T-box 4
TMA	Tissue microarray
TLX3	T-cell leukemia homeobox 3
TSG	tumor suppressor gene
TRIM58	tripartite motif containing 58
ZNF154	zinc finger protein 154
ZNF702	zinc finger protein 702, pseudogene

Financial Support

B.R., O.N., P.S., and T.L. were supported by a grant of the Tumorzentrum Heidelberg/Mannheim. B.R., J.L.B., M.K., P.S., R.G., R.P., and T.L. were supported by a grant of the Deutsche Forschungsgemeinschaft (SFB/TRR77, subprojects B4, B5, Z2).

Abstract

To identify new tumor suppressor gene candidates relevant for human hepatocarcinogenesis we performed genome-wide methylation profiling and vertical integration with array-based CGH, and expression data from a cohort of well-characterized human hepatocellular carcinomas (HCC).

Bisulfite converted DNAs from 63 HCCs and 10 normal control livers were analyzed for the methylation status of more than 14,000 genes. After defining the differentially methylated genes in HCCs, we integrated their DNA copy number alterations as determined by array-CGH data and correlated them with gene expression to identify genes potentially silenced by promoter hypermethylation. Aberrant methylation of candidates was further confirmed by pyrosequencing and methylation-dependency of silencing was determined by 5-aza-dC treatment.

Methylation profiling revealed 2226 CpG sites which showed methylation differences between normal control livers and HCCs. Of these, 537 CpG sites were hypermethylated in the tumor DNA, whereas 1689 sites showed promoter hypomethylation. The hypermethylated set was enriched for genes known to be inactivated by the polycomb repressive complex 2, while the group of hypomethylated genes was enriched for imprinted genes. We identified 3 genes matching all of our selection criteria for a tumor suppressor gene (PER3, IGFALS, PROZ). Period homolog 3 (PER3) was downregulated in human HCCs compared to peritumorous and normal liver tissues. 5-aza-dC treatment restored PER3 expression in HCC cell lines indicating that promoter hypermethylation was indeed responsible for gene silencing. Additionally, functional analysis supported a tumor suppressive function for PER3 and IGFALS *in vitro*.

Conclusion: The present study illustrates that vertical integration of methylation data with high resolution genomic and transcriptomic data facilitates the identification of new tumor suppressor gene candidates in human HCC.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most frequent cancer worldwide and has a poor prognosis . Various etiologies have been linked to HCC development, most of which cause chronic liver damage and finally lead to liver cirrhosis. The most prevalent etiological factors are chronic hepatitis B (HBV) and C (HCV) virus infections, chronic alcohol consumption and, in certain geographical areas, aflatoxin B1 food contamination . About 10% of HCC patients lack viral hepatitis, alcoholic history or other defined causes, like genetic hemochromatosis or α1-antitrypsin deficiency and these so-called cryptogenic HCCs have been shown to frequently evolve from non-alcoholic steatohepatitis. While generation of reactive oxygen species has been suggested to drive hepatocarcinogenesis in HCCs of alcoholic or cryptogenic etiology, viral-associated mechanisms are complex and involve both host and viral factors.

Human hepatocarcinogenesis is considered a step-wise process in which genetic and epigenetic alterations lead to the activation of oncogenes and the inactivation of tumor suppressor genes (TSG). In contrast to genetic alterations, epigenetic changes that include aberrant methylation and histone modification do not alter the genetic information, but affect the efficacy of mRNA transcription. Altered DNA methylation pattern belongs to the hallmarks of cancer . While altered methylation has been initially assumed as a silencing mechanism for tumor suppressor genes, developmental programs, and imprinting , it is also crucial for maintaining cell identity and fate . Aberrant hypermethylation of promoter-associated CpG islands has been observed in cancer and affects genes that are involved in main cellular processes, like apoptosis, cell adhesion, DNA repair, and proliferation .

In the past DNA methylation analyses have been carried out mainly by locus specific techniques following bisulfite conversion of unmethylated cytosines .

In this study, we performed a genome wide methylation profiling of 63 HCC samples of well-defined etiologies that had been previously characterized for genomic aberrations by array-based comparative genomic hybridization (aCGH) . We used the vertical integration of epigenomic, genomic, and expression data as a strategy for the identification of tumor suppressor gene candidates in human hepatocarcinogenesis and characterized the candidate genes Period homolog 3 (PER3), insulin-like growth factor binding protein, acid labile subunit (IGFALS), and protein Z (PROZ) in cell culture.

Materials & Methods

Tumor material and patient characteristics

63 human HCCs were analyzed for genome-wide methylation changes. The HCCs included 38 liver resections and 22 explant liver specimen; median age at surgery was 57 years (range 16 to 78) and the male/female ratio was 4:1. All diagnoses were confirmed by histological re-evaluation and use of the samples was approved by the local ethics committee. From three patients two HCC nodules were included that previously showed different aCGH indicating independent tumor development. Etiology was determined as previously described . The underlying etiologies were HBV (n=11), HCV (n=15), HCV-/HBV-co-infection (n=1), alcohol (n=14), cryptogenic (n=15), genetic hemochromatosis (n=2), and two HBx-positive tumors without chronic HBV infection. The patients´ characteristics are shown in Supplementary Table 1.

Illumina Infinium methylation assay

The Infinium HumanMethylation27 BeadChip (v1.2, Illumina, San Diego, USA) was used to obtain genome-wide DNA methylation profiles across 27,578 CpG dinucleotides located in a region of 1 kb around the transcription start site of 14,495 genes. Genomic DNA was isolated as described and high molecular weight DNA was subjected to bisulfite conversion using the EZ DNA Methylation Kit (Zymo Research, Irvine, USA) according to manufacturer's instruction for the use with the Infinium bead array platform. Using 500 ng of the bisulfite converted genomic DNA converted and unconverted (methylated) sites were interrogated simultaneously by two probes, one designed against the methylated site and one against the unmethylated site, followed by a single base extension of differentially labeled fluorescent nucleotides that are used for detection. The methylation of the individual CpG sites was quantified by the ratio signal from a methylated probe relative to the sum of both methylated and unmethylated probes. This value (β) varies continuously from 0 (unmethylated) to 1 (fully methylated) and was generated by the Illumina Genome Studio software v1.0. Complete methylation data are available under (http://livercancer.de/index.php?page=supplementary-material).

Pyrosequencing

The methylation status of *PER3* was validated by pyrosequencing using the Pyromark Q24 System (Hs_PER3_01_PM PyroMark CpG Assay; Qiagen, Hilden, Germany) according to manufacturer´s protocol. Methylation values based on the Pyromark Q24 Software 2.0 (Qiagen) were compared to the array data of the CpG sites of interest (HumanGRCh37; Chr.1p36.23: 7,845,070).

Reverse transcription and polymerase chain reaction

RNA was isolated from 100 mg of snap-frozen tissue using the RNeasy Midi-Kit (Qiagen) according to the manufacturer´s instructions. One µg of total RNA from tumors and normal liver tissues (n=6) were reversely transcribed with the RevertAid™ H minus Reverse Transcriptase (Fermentas, St. Leon-Rot, Germany) and analyzed using the ABI prism 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA; Sequence Detection Software v1.2.2) with Absolute SYBR Green ROX Mix (ABgene, Epsom, United Kingdom). Calculations of efficacy, normalization, and relative quantification versus 18s rRNA were done according to published algorithms . The primer sequences are listed in Supplementary Table 2.

DNA microarray hybridization and analysis. Quality and integrity of the total RNA was controlled using an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies; Waldbronn, Germany). Two hundred ng of total RNA were applied for Cy3-labelling reaction using the one color Quick Amp Labeling protocol (Agilent Technologies). Labeled cRNA was hybridized to Agilent human 8x60k microarrays at 68°C for 16 h and scanned using the Agilent DNA Microarray Scanner. Expression values were calculated by the software package Feature Extraction 10.5.1.1. Complete data are available online (http://livercancer.de/index.php?page=supplementary-material).

Western Blotting

Tissues were homogenized using the Precellys tissue homogenizer (PeqLab Biotechnology, Erlangen, Germany) and 1x lysis buffer (Cell Signaling Technology, Danvers, USA) supplemented with 1 µM proteinase inhibitor (Serva, Heidelberg, Germany) and 1x PhosSTOP (Roche, Mannheim, Germany).

Protein lysates (100 μg) were separated by SDS-polyacrylamide gel electrophoresis (8-12%) using a Minigel apparatus (Bio-Rad, Munich, Germany) and blotted using a semidry transfer cell (Bio-Rad). PVDF Membranes were washed twice with Tris buffered saline containing 0.1% Tween-20. Immobilized proteins were incubated with primary antibodies (Supplementary Table 3), and horseradish peroxidase linked anti-mouse or rabbit secondary antibodies (1:2000, Cell Signaling Technology). Immunoblots were visualized using ECL plus (GE Healthcare, Munich, Germany).

Tissue microarrays and immunohistochemistry

A tissue microarray (TMA) containing tissue from normal livers (n=20), non-tumorous liver tissue of HCC patients (n=66), and HCCs (n=76; Supplementary Table 4) was constructed as previously described , and immunohistochemistry was performed on 5 μm sections. PER3 (antibody dilution 1:100; Acris) antigen was retrieved using citrate buffer (pH 6.1; Dako, Glostrup, Denmark). For detection the EnVision method (Dako) was used. Counterstaining was performed using hemalum. Staining was assessed using the immunoreactive score as described previously : 0, absent; 1-4, weak; 5-8, moderate; 9-12, strong expression.

Vector design

A Gateway Cloning system (Invitrogen, Darmstadt, Germany) was used for expression of PER3 (CV029774.1) and PROZ (BC074906.2; Center for Cancer Systems Biology, The ORFeome Collaboration, CCSB 51a; Harvard Medical School, Boston, MA, USA) in HCC cells. An entry vector (pDONR223) containing the target gene was used to generate the expression construct by homologous recombination with a pDEST27 vector. Recombination was carried out according to the manufacturer´s instructions. The inserts of the vectors were validated by sequencing. A pCMV-SPORT6 vector containing IGFALS (BC025681) was used as supplied (Open Biosystems, Huntsville, AL, USA). Cell transfection was performed using the FuGENE HD (Promega, Mannheim, Germany) transfection reagent according to manufacturer´s protocol. Cells were harvested 48 h after transfection.

Cell lines, 5'-aza-2'-deoxycytidine treatment, transfection and functional analyses

HuH7, PLC/PRF/5, and SNU387 cells were cultured in DMEM and RPMI medium, respectively supplemented with 10% fetal bovine serum (PAA, Pasching, Austria) and 1% penicillin–streptomycin (10 mg/ml, PAA) at 37°C (5% CO_2) and passaged every 3-4 days. Cells were plated on 6 cm dishes 24 h before treatment with 10 µM 5-aza-2'-deoxycytidine (5-aza-dc; Sigma) or DMSO as control. Media and chemicals were changed every 24 h and plates were harvested after 96 h of treatment. All transfections were performed using oligofectamine (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. The short interfering RNA (siRNA) sequences are listed in Supplementary Table 2. The final siRNA concentration used was 30 nM (Eurofins MWG Operon, Ebersberg, Germany). Cell viability (MTT-assay), apoptosis (FACS-assay), and migration (2D-scratch-assay) were determined as described previously . Clonogenicity was analyzed 7 days after seeding of 15k cells . For all cell based assays, results were obtained from six replicate wells in three independent experiments.

Data quality control and statistical analyses

CpG-specific methylation patterns revealed non-normal, highly variable distributions that motivated the use of non-parametric statistics. The present study had 90% power to detect a methylation difference of 0.35. This was determined by multiplying the sample size for a two-sample t-test (two-sided, α=0.05, 6 tumors per normal sample, common standard deviation of 0.3) by the asymptotic relative efficiency for a Wilcoxon-signed-rank test under normality (0.995). Genome-wide methylation data were first filtered according to the following criteria: a β value between 0 and 1, a detection p-value (estimated by Illumina) below 0.01 and a positive median absolute deviation of single CpG methylation values. CpG site methylation differences between HCC and normal tissue were tested by Wilcoxon-rank-sum tests and quantified by median differences with 95% confidence intervals. Candidate sites were considered differentially methylated if the false discovery rate was below 0.5% (FDR q-value <0.005). P-values for internal validation of methylation differences relied on a Wilcoxon-signed-rank test. The correlation between gene expression and promoter methylation was tested by Wilcoxon-signed-rank tests and measured by Spearman's rank correlations. P-values <0.05 were considered statistically significant. Statistical analyses were implemented using the R package (R v2.10.1; http://www.r-project.org), Bioconductor , and SPSS 19.0 (SPSS, Chicago, IL, USA). Pathway

analyses based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) and cytoband analyses were carried out using the WEBGestalt software (http://bioinfo.vanderbilt.edu/webgestalt/).

Results

Genome wide methylation profiling of human HCC

Genome wide methylation profiles were obtained from 63 HCC samples and 10 normal liver controls. 12,008 CpG sites fulfilled the quality control criteria and out of these, a total of 2,226 CpG-sites were differentially methylated (q-value <0.005) in tumors compared to normal liver samples. Among them 537 CpG-sites were hypermethylated and 1,689 CpG sites were hypomethylated in tumors. Top hypermethylated CpG sites are shown in Table 1, while Supplementary Table 5 shows all CpG sites with an absolute median methylation difference >0.35. A gene ontology analysis of hypermethylated genes revealed an enrichment of genes that are either involved in metabolic processes or that are known to be commonly altered in cancer (Supplementary Table 6). Additionally, the set of hypermethylated genes comprised 20 of 125 genes that had been previously described as silenced by the Polycomb Repressive Complex 2 (PCR2, Supplementary Table 7) . CpG sites with a median hypomethylation of at least -0.40 in HCCs are shown in Table 2. Among the hypomethylated genes (q-value <0.005) we observed an enrichment for certain chromosomal regions (1q: FDR-q =1e-06; 11p15 FDR-q =7e-05; 12p13: FDR-q =6e-07; 19q13: 1e-07; 20p13: FDR-q =0.0038; 21q22: FDR-q = 8e-10). Furthermore, genes that have been shown to be imprinted were frequently hypomethylated in human HCCs compared to normal livers (Supplementary Table 8).

In order to identify methylation differences related to tumor etiology, we carried out additional analyses comparing the methylation of four etiological subgroups (HBV, HCV, alcoholic, and cryptogenic) with normal samples. As shown in Figure 1, 81 CpG sites showed specific methylation differences in HBV-induced HCCs, while HCV-induced HCCs showed 198 exclusive differentially methylated CpG units. Supplementary Table 9 lists those 65 genes that are altered in every etiological subgroup.

Narrowing in on tumor suppressor gene candidates through vertical integration of epigenomic, genomic, and transcriptomic profiling.

Tumor suppressor genes are important gatekeepers that protect against somatic evolution of cancer. According to Knudson´s hypothesis the inactivation of tumor suppressor genes requires the inactivation of both alleles. The flow chart in Figure 2 describes the strategy for the identification of new potential tumor suppressor genes. To prioritize the 537 potential tumor suppressor genes we first considered the genomic alterations as determined previously by aCGH . For this purpose we selected all chromosomal regions showing small losses (<5 Mb) of genomic information and that were present in at least 10% of cases. These included 139 chromosomal regions. After integrating hypermethylation with genomic regions that showed genomic losses 17 candidate genes remained (Table 3). We subsequently considered gene expression as an additional selection layer. Finally, period homolog 3 (PER3), protein Z (PROZ), and the acid labile subunit of the insulin-like growth factor binding protein (IGFALS) remained as genes that showed an inverse correlation between gene expression and promoter methylation indicating that promoter hypermethylation was responsible for their silencing in human HCCs (Table 3).

Validation of *Period homolog 3* as a tumor suppressor gene candidate in human HCC.

Since genes involved in the circadian rhythms have been implicated in tumorigenesis, we wanted to independently validate the methylation and expression changes detected for *Period homolog 3* (*PER3*). As shown in Suppl. Fig. 1A an excellent correlation existed between the values obtained using the Infinium array and the pyrosequencing approach demonstrating that measurements of PER3 methylation were highly reproducible (Spearman´s rho: 0.93, p <0.001). Additionally, the silencing of *PER3* mRNA in human HCCs was confirmed by real-time RT-PCR (Spearman´s rho: 0.78, p <0.001; data not shown).

PER3 was downregulated in 3 out of 6 HCC cell lines analyzed (Fig. 4A). To confirm that promoter hypermethylation was responsible for gene silencing, we treated Hep3B, HuH7, and HepG2 cells that showed PER3 downregulation with 5-aza-dC, an inhibitor of DNA methyltransferase 1, which restored PER3 expresssion in all three cell lines (shown representatively for HuH7 cells in Suppl. Fig. 1B)

demonstrating that the PER3 downregulation was indeed due to promoter hypermethylation.

PER3 expression is downregulated in human HCC.

Next we determined whether PER3 protein expression was downregulated in human HCCs. We performed immunohistochemistry using TMA (Fig. 3). PER3 expression was detectable in all normal liver tissues (n=20). Low PER3 expression was seen in 60% of normal livers, while 35% displayed a moderate expression, and 5% showed high PER3 expression. In non-tumorous liver tissues of HCC patients (n=66), 3% displayed no detectable PER3 signal at all, whereas most samples showed either weak (61%), moderate (27%) or high (9%) expression of PER3. Of the HCCs, (n=76,), 22% did not show any, 58% weak, 17% moderate, and only 3% showed strong PER3 staining. Statistical analysis revealed significantly reduced PER3 expression in HCCs compared to non-tumorous liver tissues of HCC patients as well as compared to normal liver samples (both $p<0.01$). Additionally, we observed a significantly lower PER3 expression in HCCs with vascular invasion compared to HCCs without vascular invasion ($p<0.05$). No significant associations were found with gender, etiology, tumor size, and UICC stage ($p>0.05$).

PER3 is a tumor suppressor gene in human HCC

To test whether the newly identified tumor suppressor gene candidate was indeed functional, we transiently re-expressed PER3 in HuH7 cells. This re-expression reduced clonogenicity (0.52 ± 0.03, $p<0.01$; Fig. 4B) and overall cell viability compared to mock transfected cells (0.43 ± 0.04, $p<0.01$; Fig. 4C), while migration was not significantly affected (0.89 ± 0.04, $p>0.05$; Fig. 4F). Reduced cell viability was due to apoptosis as shown by FACS analysis (2.39-fold $\pm$ 0.002 compared to Mock transfected cells; Fig. 4D) and cleavage of Caspase 3 and PARP (Fig. 4E). This effect was associated with the phosphorylation of CHEK2. In contrast, siRNA-mediated silencing of PER3 in SNU387 cells increased cell viability (1.27 ± 0.04, $p<0.01$; Fig. 4C) and decreased apoptosis rate compared to Mock transfected cells (0.41 ± 0.03, $p<0.05$; Fig. 4D).

Functional characterization of the potential tumor suppressor genes IGFALS and PROZ in human HCC

For functional characterization IGFALS and PROZ were also transiently expressed *in vitro*. IGFALS re-expression in HuH7 cells significantly reduced overall cell viability (0.58 ± 0.05, $p<0.01$; Fig. 5A) and clonogenicity compared to mock transfected cells (0.67 ± 0.04, $p<0.01$; Fig. 5B), which was associated with apoptosis induction (2.60-fold ± 0.01; Fig. 5C). Migration was not significantly affected (1.14 ± 0.06, $p>0.05$; Fig. 5D).

In contrast, expression of PROZ in PLC/PRF/5 cells increased cell viability (1.28 ± 0.02, $p<0.01$; Fig. 5A) and clonogenicity (1.31 ± 0.04, $p<0.01$; Fig. 5A) compared to mock transfected cells, which was associated by a slightly decreased apoptosis rate (0.81-fold ± 0.04, $p<0.05$; Fig. 5C). PROZ expression had no significant effect on migration of PLC/PRF/5 cells (0.87 ± 0.06, $p>0.05$).

Discussion

On the basis of genome wide array-based profiling of a series of well-characterized human HCCs that had been previously analyzed by high-resolution aCGH , we detected etiology-dependent and independent methylation changes in human hepatocellular carcinoma that may help to improve our understanding of human hepatocarcinogenesis.

Some of the genes showing aberrant methylation in our analysis have been addressed in single locus specific analyses, such as *CDKN2A* , *RUNX3* , *HOXA9* , *DAB2IP* , and *SOCS2* . Additionally, we confirmed recent array-based methylation analysis that showed hypermethylation of *DNM3*, *FLT4*, FoxE3, *GPR25*, *GRASP*, *HOXD1*, HTR7, IGF1R, *NETO2*, NID2, *NKX6-2*, *POU4F1*, *UTF1*, *TBX4*, *TLX3*, *TRIM58*, *ZNF154*, and *ZNF702* in human HCCs . However, none of these genes, except for *TBX4*, were considered top candidates in previous analyses , most likely due to the relatively low number of cases analyzed .

Activating mutations of β-catenin (CTNNB1) have been frequently reported in human HCCs and have been associated with a chromosomal stable phenotype . Recently, using locus specific methylation analysis Nishida et al. reported significantly higher methylation in CTNNB1-mutated HCCs compared to other HCCs , a finding we could also demonstrate on a genome-wide scale (Suppl. Fig. 2) suggesting that methylation profiling may significantly contribute to a comprehensive molecular classification of human hepatocarcinogenesis.

The evolutionary highly conserved Polycomb group (PcG) proteins promote gene repression through modification of chromatin structure and form multiple Polycomb Repressive Complexes (PRC) that exert intrinsic histone methyltransferase activity and maintain methylation of core histones . PRC2 has been linked to both stem cell biology and cancer . Polycomb target gene methylation has been reported to result in a specific stem-cell like chromatin pattern through *de novo* methylation in cancer . Here we could demonstrate that PCR2 target genes are prone to promoter hypermethylation in human HCC as recently proposed by Ammerpohl et al. . Polycomb group proteins may represent interesting translational targets, since the S-adenosylhomocysteine hydrolase inhibitor 3-Deazaneplanocin A has been shown to selectively induce apoptosis in cancer cells through depletion of PRC2 components .

When comparing the hypomethylated genes with previous array-based methylation profiling approaches , two new genes showed loss of imprinting in (*CTNNA3*, *IGF2*-

AS) and the long non-coding RNA *LINC00152* was detected as differentially hypomethylated during hepatocarcinogenesis. Furthermore, the recently described phenomenon that hypomethylated promoters form cluster across the genome (chromosomes 16, 17, 19, 20, 21, 22, and X) could be confirmed and refined (e.g. to regions 19q13, 20p13, and 21q22). Additionally, new clusters of hypomethylated DNA could be identified at 1q, 11p15, and 12p13.

Our approach to profile the methylation changes in hepatocellular carcinomas and to integrate these data with preexisting aCGH and expression data revealed three TSG candidates (*IGSALS*, *PER3*, *PROZ*) of which *PER3* and *IGFALS* were validated as tumor suppressor genes in human hepatocarcinogenesis . PER3 expression was significantly lower in HCCs with vascular invasion, a negative prognostic feature . The *PER3* gene is located on chromosomal arm 1p36, which in addition showed genomic losses in 16% (139/871) of human HCCs . It belongs to the period gene family (PER) that controls circadian rhythms . The circadian clock is organized through a complex network of feedback loops that drive rhythmic expression patterns of core clock components in mammals . Furthermore, the PER family members – including PER3 - have been implicated in cell cycle control, DNA damage response, as well as tumor progression and recurrence . PER3 physically interacts with ATM and the checkpoint kinase CHEK2 and silencing of PER3 impairs CHEK2 activation following DNA damage, while its overexpression results in apoptosis via induction of CHEK2. Thus, PER3 is likely to function as a checkpoint protein relevant for checkpoint activation and apoptosis . In line with these observations our data demonstrate an induction of CHECK2 phosphorylation after PER3 re-expression indicating that the protumorigenic PER3 function in human HCC is likely to be mediated via CHEK2 (Suppl. Fig. 3).

Besides transcriptional activation due to loss of promoter-specific imprinting or re-activation of the fetal promoter pattern, dysregulation of insulin-like growth factor-II (IGF-II) signaling in HCC predominantly occurs at the level of IGF-II bioavailability. The majority of IGF-II circulates in the serum as a complex with the insulin-like growth factor binding protein (IGFBP)-3 or IGFBP-5, and an acid-labile subunit (IGFALS). The function of IGFALS is to prolong the half-life of the IGF-IGFBP-3/IGFBP-5 binary complexes . The downregulation of IGFBPs may increase the IGF-II bioavailability in HCC . Our findings following IGFALS expression in HCC cells

indicate that in addition the epigenetic silencing of IGFALS contributes to the dysregulation of IGF-II signalling in HCC.

Several hemostatic system components, including factor X, contribute to cancer progression. PROZ is a vitamin K-dependent factor that in complex with the protein Z-dependent protease inhibitor (ZPI) inhibits activated factor X on phospholipid surfaces . Although PROZ matched our selection criteria for a tumor suppressor gene, the functional analysis did not support its tumor suppressive function. In contrast, re-expression in PLC/PRF/5 cells even suggested a protumorigenic function *in vitro*. In line with our functional findings, PROZ expression has been observed in several human cancers suggesting that the PROZ/ZPI complex might support the invasion and metastasis of tumor cells .

In summary, we describe aberrant methylation profiles in human HCC and provide evidence that the integration of epigenetic alteration pattern is essential for a comprehensive classification of human hepatocarcinogenesis. Additionally, we show that the vertical integration of methylation data with high resolution genomic and transcriptomic data allows for the identification of promising tumor suppressor gene candidates in human HCC. It highlights the potential for efficient epigenetic approaches for prevention and therapy of human HCCs.

Acknowledgements

We are grateful to Verena Kautz, Sarah Meßnard, and Eva Eiteneuer for excellent technical assistance. We thank Stefan Pusch and Marcus Renner for providing the expression vector and PER3 clone, respectively.

Authors' Disclosures of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

References

1. Bosch FX, Ribes J, Borras J. Epidemiology of primary liver cancer. Semin Liver Dis 1999;19:271-285.
2. Badvie S. Hepatocellular carcinoma. Postgrad Med J 2000;76:4-11.
3. Jones PA, Baylin SB. The Epigenomics of Cancer. Cell 2007;128:683-692.
4. Margueron R, Reinberg D. The Polycomb complex PRC2 and its mark in life. Nature 2011;469:343-349.
5. Reik W, Dean W, Walter J. Epigenetic reprogramming in mammalian development. Science 2001;293:1089-1093.
6. Kim K, Doi A, Wen B, Ng K, Zhao R, Cahan P, Kim J, et al. Epigenetic memory in induced pluripotent stem cells. Nature 2010;467:285-290.
7. Gonzales KA, Ng HH. Choreographing pluripotency and cell fate with transcription factors. Biochim Biophys Acta 2011;1809:337-349.
8. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646-674.
9. Roncalli M, Bianchi P, Bruni B, Laghi L, Destro A, Di Gioia S, Gennari L, et al. Methylation framework of cell cycle gene inhibitors in cirrhosis and associated hepatocellular carcinoma. Hepatology 2002;36:427-432.
10. Nishida N, Nagasaka T, Nishimura T, Ikai I, Boland CR, Goel A. Aberrant methylation of multiple tumor suppressor genes in aging liver, chronic hepatitis, and hepatocellular carcinoma. Hepatology 2008;47:908-918.
11. Schlaeger C, Longerich T, Schiller C, Bewerunge P, Mehrabi A, Toedt G, Kleeff J, et al. Etiology-dependent molecular mechanisms in human hepatocarcinogenesis. Hepatology 2008;47:511-520.
12. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45.
13. Longerich T, Breuhahn K, Odenthal M, Petmecky K, Schirmacher P. Factors of transforming growth factor beta signalling are co-regulated in human hepatocellular carcinoma. Virchows Arch 2004;445:589-596.
14. Brauckhoff A, Malz M, Tschaharganeh D, Malek N, Weber A, Riener MO, Soll C, et al. Nuclear expression of the ubiquitin ligase seven in absentia homolog (SIAH)-1 induces proliferation and migration of liver cancer cells. J Hepatol 2011;55:1049-1057.

15. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, et al. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 2004;5:R80.
16. Schlesinger Y, Straussman R, Keshet I, Farkash S, Hecht M, Zimmerman J, Eden E, et al. Polycomb-mediated methylation on Lys27 of histone H3 pre-marks genes for de novo methylation in cancer. Nat Genet 2007;39:232-236.
17. Feng Q, Stern JE, Hawes SE, Lu H, Jiang M, Kiviat NB. DNA methylation changes in normal liver tissues and hepatocellular carcinoma with different viral infection. Exp Mol Pathol 2010;88:287-292.
18. Calvisi DF, Ladu S, Conner EA, Seo D, Hsieh JT, Factor VM, Thorgeirsson SS. Inactivation of Ras GTPase-activating proteins promotes unrestrained activity of wild-type Ras in human liver cancer. J Hepatol 2011;54:311-319.
19. Martinez-Chantar ML, Vazquez-Chantada M, Ariz U, Martinez N, Varela M, Luka Z, Capdevila A, et al. Loss of the glycine N-methyltransferase gene leads to steatosis and hepatocellular carcinoma in mice. Hepatology 2008;47:1191-1199.
20. Gao W, Kondo Y, Shen L, Shimizu Y, Sano T, Yamao K, Natsume A, et al. Variable DNA methylation patterns associated with progression of disease in hepatocellular carcinomas. Carcinogenesis 2008;29:1901-1910.
21. Hernandez-Vargas H, Lambert MP, Le Calvez-Kelm F, Gouysse G, McKay-Chopin S, Tavtigian SV, Scoazec JY, et al. Hepatocellular carcinoma displays distinct DNA methylation signatures with potential as clinical predictors. PLoS ONE 2010;5:e9749.
22. Ammerpohl O, Pratschke J, Schafmayer C, Haake A, Faber W, von Kampen O, Brosch M, et al. Distinct DNA methylation patterns in cirrhotic liver and hepatocellular carcinoma. Int J Cancer 2011.
23. Tao R, Li J, Xin J, Wu J, Guo J, Zhang L, Jiang L, et al. Methylation profile of single hepatocytes derived from hepatitis B virus-related hepatocellular carcinoma. PLoS ONE 2011;6:e19862.
24. Shen J, Wang S, Zhang YJ, Kappil M, Wu HC, Kibriya MG, Wang Q, et al. Genome-wide DNA methylation profiles in hepatocellular carcinoma. Hepatology 2012.
25. Prange W, Breuhahn K, Fischer F, Zilkens C, Pietsch T, Petmecky K, Eilers R, et al. Beta-catenin accumulation in the progression of human hepatocarcinogenesis

correlates with loss of E-cadherin and accumulation of p53, but not with expression of conventional WNT-1 target genes. J Pathol 2003;201:250-259.

26. Laurent-Puig P, Legoix P, Bluteau O, Belghiti J, Franco D, Binot F, Monges G, et al. Genetic alterations associated with hepatocellular carcinomas define distinct pathways of hepatocarcinogenesis. Gastroenterology 2001;120:1763-1773.

27. Boyault S, Rickman DS, de Reynies A, Balabaud C, Rebouissou S, Jeannot E, Herault A, et al. Transcriptome classification of HCC is related to gene alterations and to new therapeutic targets. Hepatology 2007;45:42-52.

28. Nishida N, Nishimura T, Nagasaka T, Ikai I, Goel A, Boland CR. Extensive methylation is associated with beta-catenin mutations in hepatocellular carcinoma: evidence for two distinct pathways of human hepatocarcinogenesis. Cancer Res 2007;67:4586-4594.

29. Cao R, Wang L, Wang H, Xia L, Erdjument-Bromage H, Tempst P, Jones RS, et al. Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science 2002;298:1039-1043.

30. Muller J, Hart CM, Francis NJ, Vargas ML, Sengupta A, Wild B, Miller EL, et al. Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. Cell 2002;111:197-208.

31. Boyer LA, Plath K, Zeitlinger J, Brambrink T, Medeiros LA, Lee TI, Levine SS, et al. Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature 2006;441:349-353.

32. Gil J, Bernard D, Peters G. Role of polycomb group proteins in stem cell self-renewal and cancer. DNA Cell Biol 2005;24:117-125.

33. Widschwendter M, Fiegl H, Egle D, Mueller-Holzner E, Spizzo G, Marth C, Weisenberger DJ, et al. Epigenetic stem cell signature in cancer. Nat Genet 2007;39:157-158.

34. Tan J, Yang X, Zhuang L, Jiang X, Chen W, Lee PL, Karuturi RK, et al. Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. Genes Dev 2007;21:1050-1063.

35. Lu CY, Hsieh SY, Lu YJ, Wu CS, Chen LC, Lo SJ, Wu CT, et al. Aberrant DNA methylation profile and frequent methylation of KLK10 and OXGR1 genes in hepatocellular carcinoma. Genes Chromosomes Cancer 2009;48:1057-1068.

36. Okamura Y, Nomoto S, Kanda M, Hayashi M, Nishikawa Y, Fujii T, Sugimoto H, et al. Reduced expression of reelin (RELN) gene is associated with high recurrence rate of hepatocellular carcinoma. Ann Surg Oncol 2011;18:572-579.
37. Stefanska B, Huang J, Bhattacharyya B, Suderman M, Hallett M, Han ZG, Szyf M. Definition of the landscape of promoter DNA hypomethylation in liver cancer. Cancer Res 2011;71:5891-5903.
38. Lin YM, Chang JH, Yeh KT, Yang MY, Liu TC, Lin SF, Su WW, et al. Disturbance of circadian gene expression in hepatocellular carcinoma. Mol Carcinog 2008;47:925-933.
39. McHugh PP, Gilbert J, Vera S, Koch A, Ranjan D, Gedaly R. Alpha-fetoprotein and tumour size are associated with microvascular invasion in explanted livers of patients undergoing transplantation with hepatocellular carcinoma. HPB (Oxford) 2010;12:56-61.
40. Longerich T, Mueller MM, Breuhahn K, Schirmacher P, Benner A, Heiss C. Oncogenetic tree modeling of human hepatocarcinogenesis. Int J Cancer 2012;130:575-583.
41. Tei H, Okamura H, Shigeyoshi Y, Fukuhara C, Ozawa R, Hirose M, Sakaki Y. Circadian oscillation of a mammalian homologue of the Drosophila period gene. Nature 1997;389:512-516.
42. Ko CH, Takahashi JS. Molecular components of the mammalian circadian clock. Hum Mol Genet 2006;15 Spec No 2:R271-277.
43. Chen ST, Choo KB, Hou MF, Yeh KT, Kuo SJ, Chang JG. Deregulated expression of the PER1, PER2 and PER3 genes in breast cancers. Carcinogenesis 2005;26:1241-1246.
44. Hunt T, Sassone-Corsi P. Riding tandem: circadian clocks and the cell cycle. Cell 2007;129:461-464.
45. Gery S, Komatsu N, Baldjyan L, Yu A, Koo D, Koeffler HP. The circadian gene per1 plays an important role in cell growth and DNA damage control in human cancer cells. Mol Cell 2006;22:375-382.
46. Climent J, Perez-Losada J, Quigley DA, Kim IJ, Delrosario R, Jen KY, Bosch A, et al. Deletion of the PER3 gene on chromosome 1p36 in recurrent ER-positive breast cancer. J Clin Oncol 2010;28:3770-3778.
47. Im JS, Jung BH, Kim SE, Lee KH, Lee JK. Per3, a circadian gene, is required for Chk2 activation in human cells. FEBS Lett 2010;584:4731-4734.

48. Domene HM, Hwa V, Argente J, Wit JM, Camacho-Hubner C, Jasper HG, Pozo J, et al. Human acid-labile subunit deficiency: clinical, endocrine and metabolic consequences. Horm Res 2009;72:129-141.
49. Breuhahn K, Longerich T, Schirmacher P. Dysregulation of growth factor signaling in human hepatocellular carcinoma. Oncogene 2006;25:3787-3800.
50. Vasse M. The protein Z/protein Z-dependent protease inhibitor complex. Systemic or local control of coagulation? Hamostaseologie 2011;31:155-158, 160-154.