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Identification of HERC5 and its potential role in NSCLC progression

Michaela Wrage[1]*, Wolfgang Hagmann[2]*, Dirk Kemming[3], Faik G. Uzunoglu[4], Sabine Riethdorf[1], Katharina Effenberger[1], Manfred Westphal[5], Katrin Lamszus[5], Soo-Zin Kim[2], Natalia Becker[6], Jakob R. Izbicki[4], Juan Sandoval[7], Manel Esteller[7,8,9], Klaus Pantel[1], Angela Risch[2,10] and Harriet Wikman[1]

[1] Institute of Tumor Biology, University Medical Center Hamburg-Eppendorf, Hamburg, 20246, Germany
[2] Division of Epigenomics and Cancer Risk Factors, DKFZ - German Cancer Research Center, Heidelberg, Germany
[3] European Laboratory Association, Ibbenbueren, Germany
[4] Institute of Neuropathology, University Medical Center Hamburg-Eppendorf, Hamburg, 20246, Germany
[5] Department of General, Visceral and Thoracic Surgery University Medical Center Hamburg-Eppendorf, Hamburg, 20246, Germany
[6] Division of Biostatistics, DKFZ - German Cancer Research Center, Heidelberg, Germany
[7] Cancer Epigenetics and Biology Program (PEBC), Bellvitge Biomedical Research Institute (IDIBELL), 08908 Barcelona, Catalonia, Spain
[8] Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain
[9] Department of Physiological Sciences II, School of Medicine, University of Barcelona, Barcelona, Spain
[10] Translational Lung Research Center Heidelberg (TLRC-H), Member of the German Center for Lung Research (DZL), Heidelberg, Germany

For better lung cancer diagnosis and therapy, early detection markers of tumor dissemination are urgently needed, as most lung cancers do not show symptoms until extensive metastasis formation has already taken place. Our previous studies showed that in non-small cell lung cancer (NSCLC) early tumor dissemination is associated with a loss of chromosome 4q12-q32 and the presence of disseminated tumor cells (DTC) in the bone marrow. In order to identify the potential target gene in this region, a screen for methylation-dependent expression was performed. Lung cancer cell lines showing a loss of 4q as well as a normal bronchial epithelial cell line as control were treated with 5-aza-2'-deoxycytidine (5-aza-CdR) followed by expression profiling. Seven genes within the 4q target region, which have been associated with a positive DTC status before were found to be regulated by hypermethylation. QRT-PCR in an independent sample set identified *HERC5* as a potential target gene. Quantitative methylation analysis of these lung tissue samples revealed that *HERC5* promoter hypermethylation was significantly associated with positive DTC status ($p = 0.020$) and occurrence of brain metastases ($p = 0.015$). In addition, hypermethylation of the *HERC5* promoter in NSCLC was identified as a predictor for poor survival for Stage I adenocarcinoma patients ($p = 0.022$) and also for poor overall survival in metastatic lung cancer patients ($p = 0.028$). In conclusion, *HERC5* may function as a prognostic marker and is associated with tumor dissemination in lung cancer.

Key words: lung cancer, micrometastasis, DTC, methylation, arrays, HERC5

Additional Supporting Information may be found in the online version of this article.

Abbreviations: ADC: adenocarcinoma; 5-aza-CdR: 5-aza-2′-deoxycytidine; BM: bone marrow; BrMET: brain metastases; CGI; CpG island; DTC: disseminated tumor cells; NSCLC: non-small cell lung cancer; LCLC: large cell lung cancer; MSG: metastasis suppressor gene; PT: primary tumor; SCC: squamous cell carcinoma

*M. W. and W. H. contributed equally to this work

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Correspondence to: Harriet Wikman, PhD, Institute of Tumor Biology, University Medical Center Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany, Tel.: +49-40-74105 1913, E-mail: h.wikman@uke.uni-hamburg.de

Non-small cell lung cancer (NSCLC) is the leading cause of cancer deaths worldwide accounting for 25% of all cancer deaths.[1] Even after complete surgical resection of early stage primary tumors, 50% of patients will develop metastases within 5 years.[2] A major challenge in lung cancer research is thus to identify those patients at high risk for recurrence after surgical resection, as well as patients who would benefit from adjuvant or more aggressive treatment. Numerous molecular studies, such as those involving gene expression profiling, have shown that various inherent and acquired genetic alterations influence prognosis.[3]

Single disseminated tumor cells (DTCs) may spread to distant organs via the blood stream (hematogenous spread) or invade into regional lymph nodes via the lymphatic vessels (lymphogenous spread). The presence of these single cells found, for example, in the bone marrow (BM) has been shown to be an independent prognostic factor for survival, especially for breast cancer patients.[4] In NSCLC, the prognostic value of DTCs have shown controversial results.[5–7] However, we have recently identified specific molecular patterns

What's new?

In order to improve the diagnosis and therapy of lung cancer, early biomarkers of tumor dissemination are urgently needed. In this study, the authors found that the *HERC5* gene on chromosome 4 may be involved in regulating the spread of non-small cell lung cancer (NSCLC) tumors. In cases where the promoter region of *HERC5* was hypermethylated, the number of disseminated tumor cells (DTC) and metastases increased, and survival decreased. *HERC5* may thus be a new "metastasis-suppressor" gene, and its methylation and expression status may provide prognostic biomarkers for NSCLC.

associated with the presence of DTCs in the BM in patients with primary early stage lung cancer.[8] We performed a combined expression and copy number profiling of primary lung tumors, and detected five chromosomal regions differentiating patients with or without early dissemination of the tumor cells to the BM. Heterozygotic loss of 4q12-q32 in DTC-positive NSCLC patients was the most prominent finding. The 4q deleted region spanned over 107.1 Mbp and contained 73 differentially expressed genes.[8] Interestingly, the same loss was also found to be common in brain metastases from lung cancer patients, which indicates that early hematogenous dissemination of tumor cells appears to be a specific process driven by a defined set of molecular changes. Furthermore, allelic imbalance analyses and FISH studies identified the core region 4q21.2–22.1, which was significantly associated with worse prognosis. In addition, the same loss could be identified in single DTCs, pinpointing the importance of this region in metastasis.[9]

In other tumor entities such as colorectal, pancreatic and hepatocellular cancer loss of 4q has also been associated with worse prognosis or advanced disease stage.[10–12] In addition, in NSCLC loss of 4q has previously been associated with metastatic disease.[13] Several potential tumor suppressor genes on 4q have been suggested for different cancer types, but none of them has so far been proven to be a *bona fide* metastasis suppressor gene (MSG) in lung cancer.[11,14,15] By definition, MSGs do not influence tumor growth at the primary site, but control the capacity to escape from the primary tumor and to form overt metastases at distant sites. In contrast to classical tumor suppressor genes, MSGs seem to be rarely mutated and thus epigenetic events, such as methylation, may represent the main mechanisms of their loss of function.[16] In this study, we try to identify the possible target genes on 4q responsible for the early micrometastatic spread of lung cancer. To narrow down the potential MSG in this region, a methylation-dependent expression array-screening was performed using lung cancer cell lines with a 4q loss. QRT-PCR and methylation analyses on tissue samples from normal lung tissue and primary lung tumors as well as from brain metastases identified *HERC5* as a potential MSG gene localized on the 4q22 region. Finally, the hypermethylation at the *HERC5* promoter site in tumor tissue was also identified as a predictor for poor overall survival (OS) in Stage I adenocarcinomas (ADC), and also for poor OS in metastatic lung cancer patients (Stage IV) of all histological subtypes.

Methods

Patient and tissue samples

Two different sample cohorts were used in this study. For the first screening and evaluation of genes silenced by promoter methylation using qRT-PCR and High Resolution Quantitative Methylation Analysis fresh-frozen primary tumor samples and brain metastases of the lung were collected from patients with either ADC, squamous (SCC) or large cell lung cancer (LCLC) that underwent surgical resection at the Central Hospital Gauting, München, or the University Medical Center, Hamburg-Eppendorf, Germany. This study received ethics review board approval and sample donors gave written informed consent. Clinical data are summarized in Supporting Information Table 1. Nucleic acid extraction from primary tumors as well as brain metastases and DTC analysis was performed as described previously.[8]

In the following step, an independent sample cohort of lung tumors was used for validation of the potential impact of HERC5 methylation on OS in lung cancer patients. The used FP7 Curelung discovery cohort consists of cancer tissue of 198 surgically resected NSCLC patients. The clinical characteristics for these NSCLC patients are provided in Supporting Information Table 2 and corresponding methylation data is available in our previous study.[17]

Fluorescence *in-situ hybridization*

Fluorescence *in-situ* hybridization (FISH) analyses on the four bronchial cell lines (Calu-6, H1993, H1395 and BEAS-2B) for detecting copy number changes at 4q21 were performed using the BAC probe RP11–570L13 (RZPD, Berlin, Germany) as described before.[8] The BAC DNA was isolated using the Large Construct Kit (Qiagen) and labeled by random priming with d-UTP labeled with spectrum orange using the BioPrime Labeling System (Invitrogen).[18] Centromer probes (CEP10 and 11) were used as reference (Vysis; Downers Grove, IL). In the case of at least two evaluable centromeres, the mean of the centromer average ratio was calculated. 100 cells of each cell line were analyzed to create the average ratio. Cell lines containing a signal-to-centromere ratio ≥ 1.5 were considered to carry a gain, those with a ratio of < 0.75 a loss.

Cell Lines and 5-aza-CdR treatment

The simian virus 40 (SV40)-transformed human bronchial epithelial cell line BEAS-2B was cultured in BEBM medium

including BEBM Single Quots supplements (Clonetics, Walkersvill, MD) (kind gift from Dr. Norppa, Finish Institute of Occupational Health, Finland).[19] The human lung cancer cell lines H1993 and H1395 (kind gift from Dr. Müller-Tidow, University Hospital Halle, Germany) were grown in RPMI-1640 medium (Life Technologies) supplemented with 10% fetal bovine serum and 2 mM glutamine (Gibco). Calu-6 (HTB-56; CLS Cell Lines Service GmbH, Germany) was cultured in MEM medium containing 10% fetal bovine serum, 2 mM glutamine, 1% sodium pyruvate (Gibco) and 1% essential amino acids (Gibco). All cell lines were grown in a humidified atmosphere with 5% CO_2 at 37°C. The BEAS-2B, H1993, and H1395 cell lines were authenticated using Multiplex Cell Authentication (Multiplexion, Heidelberg, Germany) as described recently.[20]

5-aza-2'-deoxycytidine (5-aza-CdR; Sigma, St. Louis, MO) was solubilized in water. First the optimal 5-aza-CdR concentration was tested, in order to achieve an optimal conversion of all methylated cytosines but with minimum cellular stress. Therefore, cells were cultured in growth medium, conditioned with 0.5, 1 or 5 μM 5-aza-CdR and exchanged daily until total confluence was reached after 5 to 7 days. For the final array experiments every cell line was plated with an initial cell confluence of ∼15% and cultured for 6 hr before 1 μM 5-aza-CdR treatment. All experiments were performed in triplicates using different cell passages of cells. The cells were grown until total confluence and the growth medium containing 1 μM 5-aza-CdR was exchanged every day. The nucleic acid extraction from both untreated and treated cell lines was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) including DNase I treatment for RNA extraction. Sodium bisulfite sequencing as well as qRT-PCR of genes known to be methylated (*MAGE1*, *MAGE12*, *GSTP1*) were used as verification of successful 5-aza-CdR treatment.

In addition, by using MALDI-TOF mass spectrometry with the EpiTYPER application (Sequenom, San Diego, CA)[21,22] we quantitatively measured DNA methylation levels at two genomic loci (long interspersed nuclear element 1 (*LINE1*) and *HERC5*) in three lung cell lines (H1993, H1395 and Calu-6) treated with or without 1.0 μM 5-aza-CdR. We chose *LINE1* repeat DNA methylation as a surrogate to verify the effectiveness of demethylation by 5-aza-CdR treatment.

Analysis of methylation-dependent expression in cell lines

Equal amounts of RNA (500 ng) from 5-aza-CdR and nontreated cell lines were cohybridized on 4x44 K human Two-Color Gene Expression Microarrays (Agilent Technologies).[24] In brief, 500 ng RNA from untreated or 5-aza-CdR treated cell lines each from three independent experiments was pooled and labeled with Cy5-dCTPs (untreated) or Cy3-dCTPs (5-aza-CdR treated) using the Quick Amp Labeling Kit, two-color (Agilent Technologies) and hybridized on an array. The arrays were median normalized and log transformed. The MIAME guidelines were followed in sample, array and data processing. Genes deemed to have their expression potentially regulated by methylation were defined as showing a >2-fold expression difference between the treated and nontreated cells in at least one tumor cell line. To restrict analyses to genes specifically deregulated in tumors another requirement was that no difference should have been observed in the control cell line BEAS-2B. All expression data are freely available at ArrayExpress accession no. GSE57492.

Quantitative real-time RT-PCR analysis

Reverse transcription (RT) for cDNA synthesis was performed on 250 ng tumor or 1 μg total cell line RNA using the First Strand cDNA Synthesis with oligo(dT) (Invitrogen, Karlsruhe, Germany), respectively. Quantitative real-time RT-PCR (qRT-PCR) reactions were run in triplicates and performed on the Mastercycler Eppendorf Realplex. The housekeeping gene *RPLP0* (ribosomal protein, large, P0) served as internal control to confirm the success of the RT reaction. Primer sequences are listed in Supporting Information Table 3. PCR cycling conditions were 94°C (5 min) for one cycle, 94°C (15 s), 55–62°C (30 s) and 72°C (30 s) for 30 cycles, and a final extension of 72°C (5 min) (Supporting Information Table 3). Transcript abundance of the target genes and RPLP0 was measured during real-time qRT-PCR using SYBR green as fluorochrome. Gene expression was calculated using the ΔΔCT method and normalized to RPLP0 expression. The results, expressed as N-fold differences in target gene expression, were set in relation to pooled RNA from normal bronchial epithelial cells.

Sodium bisulfite conversion and high resolution quantitative methylation analysis

Genomic DNA was isolated from human primary and metastatic lung cancer, brain metastases or adjacent normal lung tissue as described previously[8] and sodium bisulfite-converted with the EZ DNA Methylation Kit (Zymo Research, Irvine, CA). Quantitative DNA methylation analyses were performed using the MassCleave assay including PCR, *in vitro* transcription, base-specific RNA cleavage and MALDI-TOF mass spectrometry with the EpiTYPER application (Sequenom, San Diego, CA) whereby the methylation status of single or neighboring CpGs (CpG units) was quantified as described previously.[23,25] Details of the primers used in these methylation analyses of the *HERC5* promoter are given in Supporting Information Table 3. To correct for a potential PCR bias and to ascertain the full dynamic range of our measurements (0–100% methylation), we included for each amplicon a six-point mix of human whole-genome amplified and fully methylated DNA standard representing 0, 20, 40, 60, 80, 100% methylated DNA and corrected the raw sample data according to best fitting curves computed with R statistical environment on http://www.r-project.org (R 2.9.0) as reported.[23]

Statistical analysis

A correlation between gene expression levels and DTC status was analyzed using the two-tailed *t*-test. An association

between the methylation status and DTC status was analyzed by the Fisher's exact test for small sample numbers. *P* values lower than 0.05 were considered statistically significant.

The differences between mean methylation of tumor samples (primary and metastatic lung cancer, brain metastases) and normal lung tissue samples determined by MassCleave assay were tested using Wilcoxon rank sum test with continuity correction. The Bonferroni-Holm correction of the *p*-values was applied to adjust for multiple testing. The evaluation of the DNA methylation status using the Infinium 450k Human BeadChip methylation array was performed as reported.[17]

The Kaplan–Meier method was used to estimate OS, and differences among the groups were analyzed with the log-rank test. Multivariate Cox proportional hazards regression was used to evaluate independent prognostic factors associated with survival. Age, gender, smoking history, histological type and tumor stage were included as covariates.

Results

DNA copy number loss of 4q in lung cell lines

Previously, we could show that a heterozygous loss of 4q in primary NSCLC correlated significantly with the presence of DTCs in the bone marrow.[8] For a complete gene repression also the second allele has to be silenced, for example, via hypermethylation of its promoter. For the methylation array analyses three different lung cancer cell lines were chosen that reflect the situation in the primary tumors, that is, showing a one-allele loss of 4q. Validation of 4q21 loss was done by FISH using the probe already used in our previous studies.[8,9]

The NSCLC cell lines, H1395, H1993 and Calu-6 showed a loss of at least one allele, whereas the reference cell line BEAS-2B showed a diploid phenotype (average: 46 chromosomes; range 45–48) with two alleles of chromosome 4q in >95% of the cells (Supporting Information Fig. 1). All of the tumor cell lines were characterized by different levels of polyploidy: H1395: hypotriploid (average: 57 chromosomes; range 54–59); H1993: tetraploid (average: 86 chromosomes; range 79–92); Calu-6: hypotriploid (average: 56 chromosomes; range 55–58).

5-Aza-CdR treatment and profiling of methylation-dependent expression in lung cell lines

To identify potential target genes on 4q silenced by promoter methylation, we measured the global gene expression changes after 5-aza-CdR treatment in three established NSCLC cell lines harboring a heterozygous loss of 4q. First the optimal 5-aza-CdR concentration was tested, in order to produce a maximal conversion of methylated cytosines but with minimum cellular stress. Three genes *MAGE1*, *MAGE12* (for BEAS-2B) and *GSTP1* (for the three NSCLC cell lines) known to be silenced by methylation, were used as controls for a successful demethylation by 5-aza-CdR.[26] 1 μM of 5-aza-CdR was shown to be an optimal concentration for conversion of methylated cytosines without significant loss of cellular viability. In addition, quantitative methylation analyses of *LINE1* loci using MALDI-TOF mass spectrometry with the EpiTYPER application verified the successful but varying degree of demethylation at these loci following 1 μM 5-aza-CdR treatment (Supporting Information Fig. 2*a*).

Gene expression changes between treated and untreated cell lines were then determined using comparative hybridization onto expression microarrays. All together 7,702 transcripts on the array were found > twofold upregulated following 5-aza-CdR treatment in at least one cell line; of these, 4395 array transcripts were exclusively found in the cancer cell lines (data not show). Within 4q, 167 transcripts representing 116 annotated genes were found twofold upregulated in at least one of the cancer cell lines (Supporting Information Table 4). Of these genes, 34 were also upregulated in BEAS-2B and where thus excluded from the downstream analysis. One gene was upregulated in all three cancer cell-lines, 11 were deregulated in two cell lines and 70 in one of the cell lines (Fig. 1*a*).

Gene expression screening for methylation-related genes

Our previous gene expression profiling of primary lung tumors with and without DTCs in the BM identified 73 differentially expressed genes on 4q12–32.1.[8] Nine of these genes were also identified in the methylation-dependent expression screening, and seven of which were exclusively deregulated in the NSCLC cell lines but not in BEAS-2B cells. *HERC5* (4q22.1), cysteine-rich hydrophobic domain 2 (*CHIC2*; 4q12) and dual adaptor of phosphotyrosine and 3-phosphoinositides (*DAPP1*; 4q23) were deregulated in two of the cell lines. Annexin A3 (*ANXA3*; 4q21.21), protein phosphatase 1K (*PPM1K*, 4q22.1), tripartite motif containing 2 (*TRIM2*; 4q31.1), and ligand of numb-protein X 1 (*LNX1*; 4q12) were all found deregulated in one NSCLC cell line each.

Quantitative real-time RT-PCR validation

The expression of the seven genes found significantly downregulated in lung tumor tissue and potentially regulated by methylation was analyzed by qRT-PCR in primary tumor samples with known DTC status (21 DTC negative, 12 DTC positive patients) and in normal peripheral lung tissues ($n = 4$). *CHIC2, DAPP1*, and *HERC5* were downregulated (>2-fold) in 70%, 74% and 61% of the tumor samples respectively, compared to the normal peripheral lung tissue. *LNX1, TRIM2, ANXA3,* and *PPM1K* were downregulated in 46, 22, 53 and 22% of the tumor samples (data not shown). When the patients were divided according to the DTC status a significant association was only found for *HERC5* ($p = 0.042$) and a borderline significance for *LNX1* ($p = 0.052$; Fig. 1*b*).

The expression of *HERC5* was also examined by qRT-PCR in the four different lung cell lines. *HERC5* was 20-fold induced after the 5-aza-CdR treatment in H1395 cells,

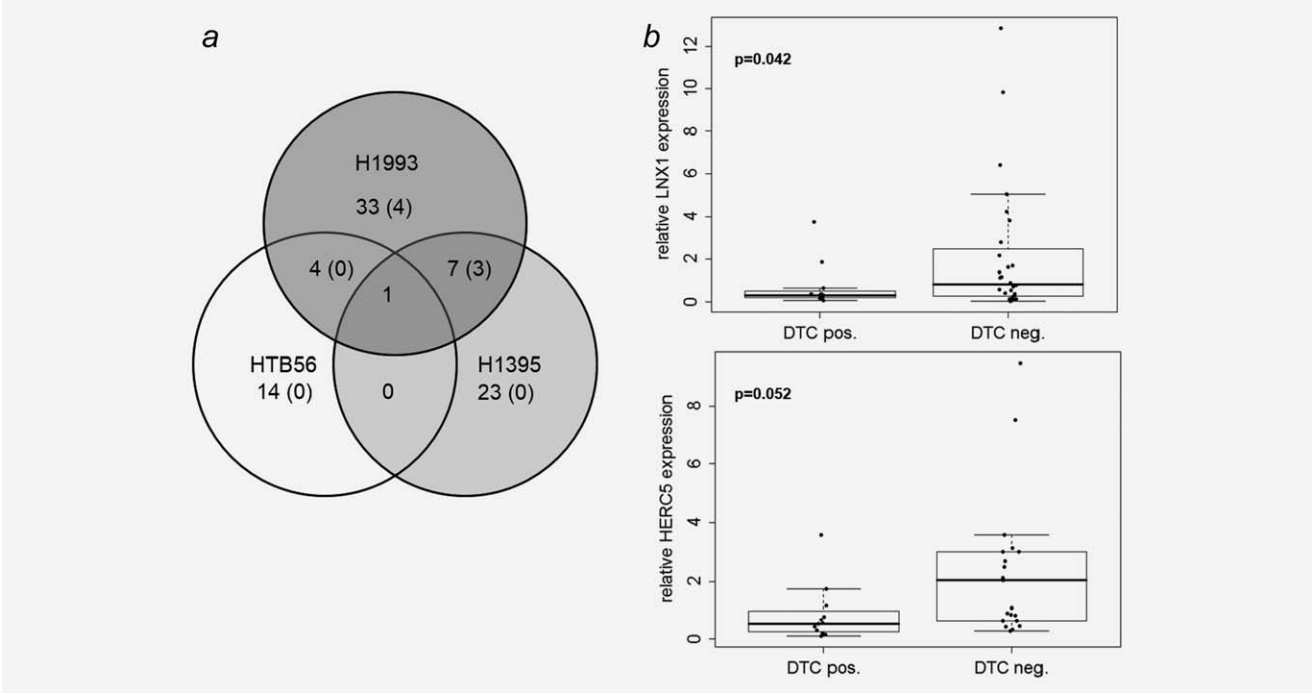

Figure 1. Genes on 4q Differentially Expressed in 5-Aza-CdR-treated and Untreated Lung Cancer Cell Lines. (*a*) Venn Diagram of the methylation-dependent expression array results. Three different NSCLC cell lines with a loss of 4q were treated with and without 5-Aza-CdR in order find genes downregulated through methylation. The values without brackets indicate the number of genes found significantly upregulated in each cell line after 5-Aza-CdR treatment. The values in brackets give the number of those genes also found downregulated in patients with DTCs in the BM compared to primary tumors without DTC. (*b*) Quantitative real-time RT-PCR results for *HERC5* and *LNX1* expression in 12 DTC positive and 21 DTC negative primary tumor patients. Relative transcript levels were determined by normalization to the reference gene *RPLP0* and normal lung tissue using the ΔΔCt method.

whereas no induction and a high endogenous level were detected in BEAS-2B cells. The H1993 cells showed an almost sixfold induction and Calu-6 cells a twofold induction of *HERC5* expression after 5-aza–CdR treatment (data not shown).

Quantitative methylation analysis

To investigate the methylation status at the *HERC5* promoter CpG island (CGI) in human primary and metastatic lung cancer, 62 samples of primary lung tumors, 20 brain metastases (BrMET) and adjacent normal lung tissue from 6–17 patients (depending on amplicon) were analyzed by MassCleave assay. Among the primary tumor cases nine showed DTCs in the BM and 53 did not show DTCs in the BM. The normal samples were matched pairs of the primary tumor tissue samples.

To detect the most informative region of methylation the three different lung cancer cell lines were investigated for the level of methylation in the *HERC5* promoter CGI by MassCleave assay. This CGI (Bp position: Chr4: 89378224–89378948) and part of the upstream sequence consisting of more than 90 CpGs was analyzed, covered by three different amplicons (HERC5 Amplicon 1, Amplicon 2 and Amplicon 3). The level of cytosine methylation at these CpGs was assessed and compared to the expression level of *HERC5* in the different cancer cell lines (Fig. 2*a*). The second amplicon (Amplicon 2; Bp position: Chr4: 89378771–89379109) showed the best correlation to the expression, and was also shown to be accessible for demethylation by 5-aza–CdR treatment (Supporting Information Fig. 2*b*). For Amplicon 2, a high average methylation was found in H1395 cells (97%; "homozygous" methylation), and partial methylation was found in H1993 (66%) and Calu-6 (55%; "heterozygous" methylation) cells. Whereas Amplicon 1 (Bp position: Chr4: 89378260–89378704) showed almost no methylation in any of the cancer cell lines (mean methylation 4–9%), the most proximal amplicon (amp 3; Bp position: Chr4: 89379135–89379442) showed a high level of methylation (50–100%) in all cancer cell lines.

For the patient samples, the methylation status was also quantified for all three amplicons (Fig. 2*b*). Like in the cancer cell lines a low methylation level was found in Amplicon 1, and consistently higher methylation in Amplicon 3. No difference between the tumor groups could be found for these amplicons.

For Amplicon 2 the degree of methylation of a total of 21 CpGs contained in 14 informative CpG units could be analyzed. The mean methylation of the CpGs in this *HERC5* promoter segment amounted to 18.5% (range: 0–97.3%) in the primary lung cancer cases, whereas only 5.5% mean

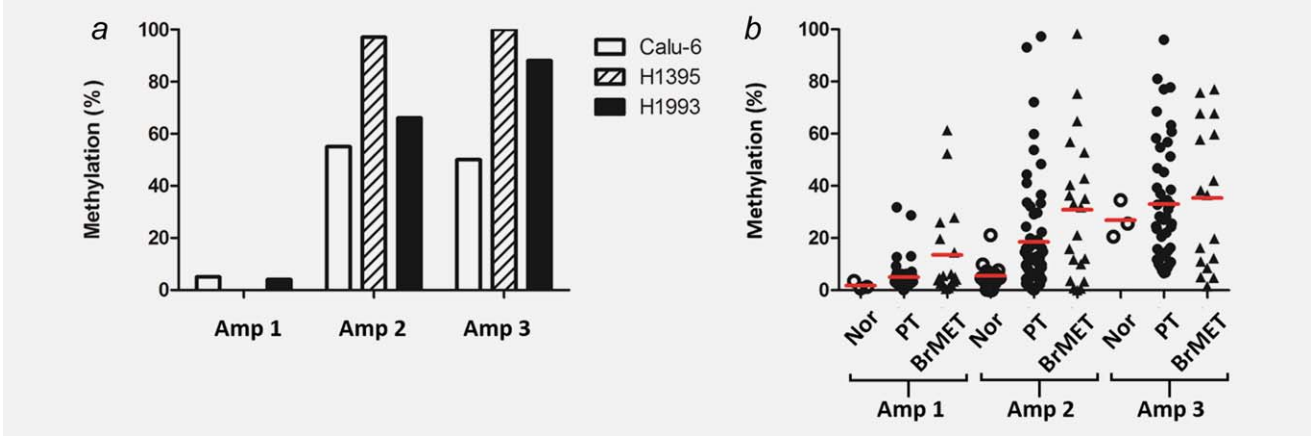

Figure 2. Methylation at *HERC5* Promoter. Data points show quantitative measurements of methylation (MassCleave assay) in *HERC5* amplicons 1, 2 and 3 in cell lines (*a*) and in patient samples (*b*). Methylation values represent average % methylation of all analyzed CpGs/amplicon, horizontal bars indicate median methylation of the specific histologic group. DNA samples: Normal (Nor) lung tissue ($n = 3–17$); primary lung tumor (PT, $n = 48–62$) or brain metastases (BrMET; $n = 18–20$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table 1. Methylation of HERC5 amp2 in normal and lung tumor tissue

		Heterozygote		Homozygote		Total	*p*-value
HERC-5 amp2	*n*	*N*	%	*n*	%	%	
Primary tumor							
DTC-	53	3	5.7	2	3.8	9.4	Ref.
DTC+	9	2	22.2	2	22.2	44.4	0.020
Brain Metastases	20	5	25.0	4	20.0	45.0	0.003
Normal	17	0	0	0	0	0	n.s.

methylation was found in the matched normal lung tissues (range: 0–21.1%) and 30.4% (range: 0–98.4%) was found in the brain metastases (BrMET) samples (Fig. 2*b*). The mean methylation among primary tumors with and without DTC was 31.7 and 16.3%, respectively. A statistically significant difference using Wilcoxon rank sum test was found between methylation of all primary tumors and the normal lung ($p = 0.001$), as well as DTC negative tumors with normal lung ($p = 0.002$) and DTC positive tumors ($p = 0.012$), respectively (Supporting Information Fig. 3). Methylation status was not significant associated with gender, TMN and UICC status. Methylation of the normal lung and brain metastases showed also significant correlation ($p = 0.003$); these differences stayed significant even after Bonferroni–Holm correction ($p=0.02$ and 0.003).

In 17 primary tumor cases matched pairs of adjacent normal lung was available. In 13 of these pairs only little or no methylation in Amplicon 2 of the *HERC5* promoter CpGs could be found in both tumor and normal tissue. Three primary tumor tissues had a methylation mean ranging between 32 and 34%, whereas the corresponding normal tissues showed only 5.1–6.1% methylation. One tumor sample showed an average of 97.3% CpG methylation in this amplicon, whereas in normal tissue the average methylation amounted to only 2.8%, indicating that *HERC5* promoter methylation is a tumor specific event.

To subgroup our samples, those within a methylation range of 31–55% (average standard-corrected methylation, corresponding to 45–78% methylation in tumor tissue, based on a> 70% tumor purity in samples) were defined as heterozygously methylated samples, and those showing > 56% methylation (corresponding to >80% methylation in the tumor fraction of sample) as homozygously methylated samples at this site. 44.4% of the primary tumor patients with DTC in the BM (PT DTC+) had either heterozygous or homozygous methylation of *HERC5* amp2 (Table 1). A similar frequency was seen for the brain metastases (BrMET) samples (45.0%), whereas only 9.4% of patients without DTC in primary tumor (PT DTC-) showed a mean methylation of *HERC5* amp2 above 35%. A statistically significant difference between DTC positive and negative patients could be found ($p = 0.020$) as well as between DTC negative patients and brain metastases samples ($p = 0.003$) (Table 1). The results stayed significant also when more rigorous methylation cut offs were used (40% and 70%, respectively; data not shown).

HERC5 methylation status in lung tumor tissue predicts OS

We tested whether the hypermethylation we found for the *HERC5* promoter in primary lung tumor and brain metastasis tissue could be validated in an independent cohort of samples. To this end, we tested data from a study using Infinium 450k Human BeadChip (Inf450k) analyses on

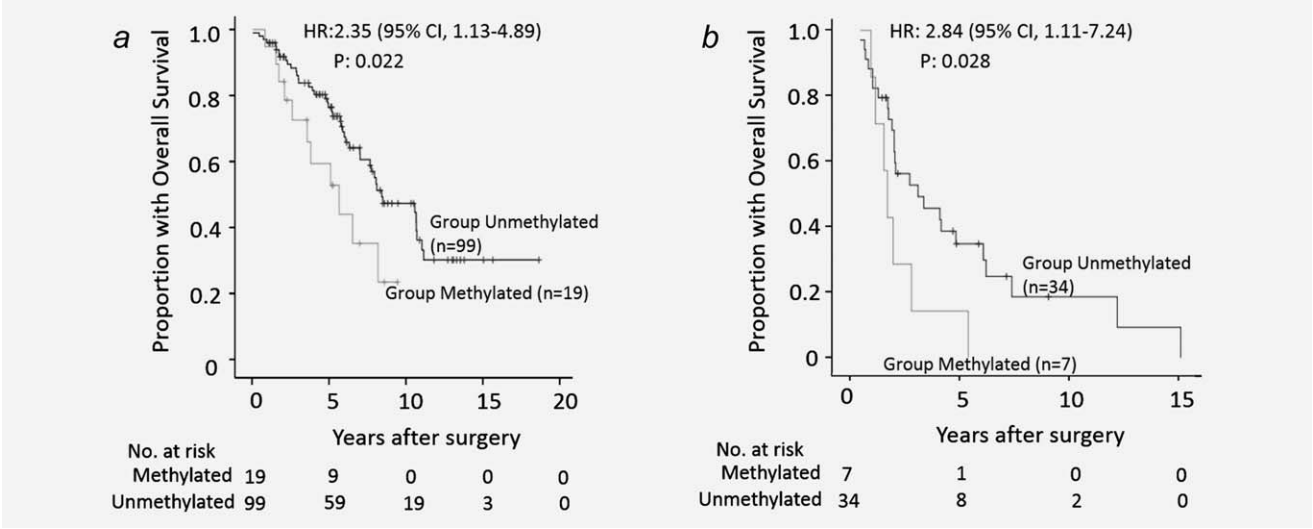

Figure 3. Kaplan–Meier analysis for OS according to methylation status of cg08750951. (*a*) Stage I and ADC non-small cell lung cancer patients ($n = 118$). (*b*) Metastatic non-small cell lung cancer patients ($n = 41$). Methylation status was determined by the Infinium 450k Human BeadChip methylation array and threshold for methylation status was set at β-value = 0.4 to define nonmethylation ($\beta < 0.4$) and methylation ($\beta > 0.4$). Log-rank test and univariate Cox regression analysis *p*-values correspond to 0.033 and 0.037 (panel A) or to 0.036 and 0.043 (panel B), respectively. The indicated *p*-value (panel A, $p = 0.022$) corresponds to the hazard ratio (HR =2.35) adjusted by multivariate Cox regression (including age, gender, smoking history and metastatic status), and the *p*-value in panel B ($p = 0.028$) corresponds to the HR of 2.84 adjusted by multivariate Cox regression (including age, gender, smoking history age, stage and histological subtype).

human lung tumor tissue[17] consisting of 198 NSCLC samples (including $n = 118$ lung Stage I ADCs and $n = 29$ Stage I SCCs). In this data set, we analyzed all four Inf450k CpGs contained within the above mentioned EpiTYPER amplicons *HERC5* amp1–3, that is, cg15471079, cg14660125, cg08750951, and cg02215171. We identified that the CpG hypermethylation status of cg08750951 from the *HERC5* gene which is identical to CpG #14 in our *HERC5* amp2 (Supporting Information Fig. 4) predicts poor OS in Stage I lung ADC patients (HR = 2.35; $p = 0.022$ and $n = 118$; Fig. 3*a*) by multivariate Cox regression, but not in Stage I lung SCC patients (HR = 0.62; $p = 0.531$, consisting of only 29) or in STAGEs II–IV of ADC (HR = 1.14; $p = 0.769$ and $n = 37$) and SCC patients (HR = 0.85; $p = 0.943$ and $n = 14$), respectively. We also found that the hypermethylation (methylation β-value > 0.4) at this cg08750951 in the *HERC5* promoter further predicts poor OS in patients who had metastases at the time of diagnosis and surgery (all Stage IV patients independent of histology) or who developed metastases during the follow-up (HR = 2.84; $p = 0.028$ and $n = 41$; Fig. 3*b*). In contrast, we did not find a significant association with any prognostic factors and methylation status for the other three Inf450K CpGs localized within the *HERC5* EpiTYPER amplicons (cg15471079, cg14660125 and cg02215171).

Discussion

Lung cancer is the most common cause of cancer related death affecting more than 1.3 million patients each year, with mortality linked to the presence of distant metastases.[1] The high risk of relapse even among early-stage lung cancer patients has been linked to the presence of DTCs in the BM at the time of operation.[5] We have previously shown that the existence of these single tumor cells in the BM is associated with a specific genetic profile with loss of 4q significantly associated with the DTC status of a patient and that loss of 4q21–22 is an independent negative prognostic factor in NSCLC.[9] However, in contrast to breast cancer where numerous large studies and meta-analyses have shown a clear correlation between DTC status and prognosis, in lung cancer the link with DTC status is controversially discussed.[7]

In this study, methylation-dependent expression screening of genes in lung cancer cell lines harboring a heterozygous 4q loss identified seven genes, which were found potentially methylated in cell lines and differentially expressed between DTC positive and negative patients in the original array data set described by Wrage *et al.*, 2009.[8] Validation of these genes in an independent data set found a significant association between *HERC5* downregulation and positive DTC status. Detailed methylation profiling of the *HERC5* promoter identified *HERC5* as a potential target MSG on 4q. Heterozygous or homozygous *HERC5* promoter methylation was detected in 44% of DTC positive patients, whereas this region was considered methylated in only 9% of the DTC negative patients. Methylation of *HERC5* was also commonly seen among brain metastases samples, whereas normal lung samples did not show any methylation at this genomic site. While the rather small number of samples in the different groups certainly asks for validation in larger cohorts, our data taken together suggest that, *HERC5* promoter methylation may serve as a prognostic factor for poor outcome or may be associated with advanced disease stage. Supporting the assumption that *HERC5* could be used as a prognostic

marker, methylation at cg08750951, using a cut-off β-value of 0.4 (40% methylation) predicts 5 year survival of Stage I ADC patients in a separate independent patient group. Interestingly *HERC5* is located within the narrow hot spot region identified in our recent study, which found loss of 4q21.2–22.1 to be an independent poor prognostic factor in NSCLC.[9] These results strongly suggest that HERC5 may be involved in the tumor dissemination of NSCLC and may qualify as a negative prognostic factor.

The gene product of HERC5 (HECT domain and RLD domain-containing protein 5; *HERC5/ceb1*), is an E3 ligase, which has been implicated to be involved in various cellular processes (reviewed in Ref. [27]. The HERC5 protein contains a homologous to E6-AP-terminus (HECT) domain and a regulator of chromosome condensation (RCC) 1-like domain (RLD).[28,29] Proteins containing both a HECT and a RCC1-like domain belong to the HERC family, which in humans consists of six members.[30]

HERC5 was first identified in a yeast two-hybrid screening as a cyclin E-p21 interaction partner and thus has been suggested to play a role in cell cycle control.[29] Interestingly, HERC5 can also specifically interact with the protein nonmetastatic cells (Nm) 23b (Nm23b/NME2).[30] NME2 is a well-described MSG involved in proliferation and differentiation.[31] The HECT domain of HERC5 binds to NME2 and conjugates an ubiquitin residue to the NME2 target, as a result of which, surprisingly, no degradation of NME2 is observed. Therefore, it was suggested that ubiquitination of NME2 serves other purposes than protein destruction.[30] HERC5 has furthermore been shown to be involved in innate immunity. Being an E3 ubiquitin ligase, HERC5 can mediate the transfer of single or multiple ISG15 conjugates to target proteins during a process called ISGylation. ISGylation is an important and well described component of interferon and NFκB-mediated innate immune response.[28] HERC5 protein function is thus associated with a variety of cellular processes including immune response mechanisms, cell cycle control and posttranslational protein modification all possibly playing an important role in metastasis.

Several MSGs have been identified lately. Few of these genes have found to be silenced by mutations, whereas silencing by methylation has been often described.[16] DNA methylation is a DNA modification of almost exclusively cytosines located within CpG dinucleotides. This chemical modification can persist through cell division, or be added or removed during the cell's lifespan, without ever changing the original DNA sequence. Due to the reversible nature of methylation, a number of demethylating agents have been subjected to an intensive investigation for their clinical use. Indeed, different inhibitors of DNA methyltransferases and histone deacetylases have shown promising antitumorigenic effects in some malignancies.[32]

The CpG dinucleotide clusters, known as CpG islands (CGIs), are often found in the promoter or first exon of many genes. For silencing of a gene, in many cases methylation of specific CpG dinucleotides is needed, particularly at CpG-rich promoter sites, whereas other regions such as intragenic parts of the genome may be methylated also in active genes. For *HERC5*, we found that the methylation state of a specific CpG locus (amp2) correlated with the expression levels of the gene, whereas other methylated CpG sites did not. Taken together, our data support the idea that silencing of the *HERC5* promoter by methylation of specific CpG sites not only correlates with HERC5 expression but also predicts poor OS of certain lung tumor patients. As this study functioned as a prospective study, future analyses have to clarify if HERC5 is directly involved in metastasis or may be act as a surrogate biomarker for other genes which promotes the metastasis pathway. Clearly, future studies will be needed to also verify the actual protein level changes of HERC5 in patients. Sadly, currently no reliable antibody is available for paraffin embedded tissue.

In conclusion, we have identified *HERC5* as a potential MSG on 4q22. Our results suggest that *HERC5* promoter methylation may be involved in early tumor dissemination and serves as a negative prognostic factor in NSCLC. The mode of action as a potential MSG needs to be investigated in future functional studies.

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