Pathol. Oncol. Res. (2015) 21:123–130
DOI 10.1007/s12253-014-9797-3

RESEARCH

Development of a *DUSP9* Methylation Screening Assay

Stefan Jenner · Klaus Herrmann Wiedorn · Dieter Techel

Received: 17 December 2013 / Accepted: 7 May 2014 / Published online: 18 May 2014

Abstract A methylation screening assay for *DUSP9* (dual-specificity phosphatase 9) has been developed and applied on 79 FFPE samples from patients with colorectal cancer (CRC) and 22 corresponding tumor free colon samples in this study. Quantitative pyrosequencing was used for the determination of the methylation in the promoter CpG island, including 83 CpG motifs. In this way, the methylation pattern of the 11 tumor samples with the weakest and the strongest methylation could be identified and were compared to their corresponding tumor free colon samples. Forty six percent of the weakly methylated samples showed no significant difference to their tumor free counterparts, whereas in 27 % of the cases an increased or reduced methylation was detectable. For the strongly methylated tumor samples only 18 % showed no significant difference to their tumor free counterparts, whereas 82 % were significantly stronger methylated. In CRC, the aberrant promoter methylation of tumor suppressor genes is one aspect that defines the CpG island methylator phenoptype (CIMP) and is frequently observed in a subpopulation of cases. Patients harboring a CIMP phenotype often show additional clinicopathological characteristics, the so called CIMP features. Interestingly, no CIMP features were found for the weakly methylated samples analyzed in this study but could be seen in 82 % of the strongly methylated cases, indicating a possible use for *DUSP9* as CIMP marker.

Keywords DUSP9 · Colorectal cancer · Promoter methylation · CIMP · Pyrosequencing

S. Jenner · K. H. Wiedorn · D. Techel
Division of Molecular Pathology, Department of Pathology, Katharinen Hospital Stuttgart, Stuttgart, Germany

S. Jenner (✉)
Institut für Pathologie, Abteilung Molekulare Pathologie, Katharinen Hospital Stuttgart, Kriegsbergstr. 60, 70174 Stuttgart, Germany
e-mail: S.Jenner@Klinikum-Stuttgart.de

Introduction

Colorectal cancer (CRC) with 1.23 million new cases and 608,000 deaths (in 2008) is one of the leading malignancies regarding incidence and mortality worldwide [1]. Over decades, scientists and clinicians studied this cancer in detail for improving the understanding of its tumorigenesis. Their discoveries include structural or numeric genomic aberrations and often these findings could also be adapted (at least in parts) to other malignancies [2, 3].

Beside sequence aberrations, scientists observe the contribution of epigenetic events in tumorigenesis, leading to the strongly growing field of epigenetic-based cancer research in the past years. Already in 1983 Feinberg and Vogelstein [4] found a remarkable methylation pattern during tumorigenesis of CRC, which could be confirmed by several further studies. In preneoplastic lesions a genome-wide hypomethylation could be detected, affecting CpG islands and repetitive sequences of chromosomes, leading to a chromosomal instability (CIN) phenotype. A de novo methylation of CpG islands occurs during tumorigenesis, affecting also the promoter regions of some specific marker genes, mainly tumor suppressor genes, and is increasing progressively from preneoplastic lesions to metastatic carcinomas [5–8]. This is resulting in an aberrant methylation pattern of these genes in tumor tissue DNA, compared to the DNA of corresponding healthy tissue. This aberrant methylation pattern of CpG motifs in CRCs is associated with a unique tumorigenesis phenotype, called CIMP (CpG island methylator phenoptype) and was first described in 1999 by Toyota et al. [9].

Toyota [9] and later Weisenberger et al. [10] postulated different sets of marker genes for the identification of CIMP in clinical CRC samples. These markers have to be stronger methylated in tumor than in the corresponding normal tissue for confirming a CIMP phenotype of the tumor. Weisenberger [10] pointed out, that at least 3 out of their 5 markers must

show a strong methylation in the tumor tissue to constitute a CIMP phenotype.

Additional to this finding, Ogino et al. [11] could correlate some clinical and molecular features to tumors with CIMP phenotype, when comparing the CIMP status of several hundred samples by using the markers of Weisenberger. Samples with a strong methylation of the CIMP markers are significantly more frequently found in proximally localized tumors from patients with female sex. Further, they are often positive for microsatellite instability (MSI) and show activating *BRAF* mutations rather than *KRAS* mutations [11–15].

The primary aim of this study was the development of a methylation screening assay for *DUSP9*. When an aberrant methylation pattern was observed, the clinical and molecular CIMP features were collected for these samples to explore a possible use of the gene as CIMP marker.

DUSP9 has been first described in 1997 by Muda and colleagues [16] and is a member of the large family of protein tyrosine phosphatases (PTPs). The *DUSP9* gene, also called MKP4 (mitogen-activated protein kinase phosphatase 4), consists of 4 Exons and has a size of 8,884 bp. It is localized on chromosome Xq28 and codes for a functional protein of 41.9 kDa.

The PTPs serve as negative regulators in different signal transduction pathways. They are involved in maintaining the balance of protein activity and are key effectors in controlling cell growth and -survival in physiological and pathological processes, including tumorigenesis [17]. While the protein tyrosine kinases (PTKs) were studied intensively over several decades, the focus on PTPs arise only a few years ago.

DUSPs dephosphorylate the MAP kinases ERK, JNK and p38. The substrate specificity varies between the different DUSPs, but the dephosphorylation always takes place at the regulatory tyrosine- or threonine-residues. Because of its key role, a misregulation of the MAPK signal transduction pathway is frequently involved in the tumorigenesis of several carcinomas and the acquisition of resistance to cancer therapies. The DUSPs are the negative regulators of diverse kinases at the end of this important pathway and therefore play a crucial role in its regulation [18–20].

While in unstimulated, adult cells the expression is reduced to a minimum, a strong expression of DUSP can be observed in stimulated cells [18, 19, 21, 22]. An aberrant regulation of *DUSP9* has already been observed in several human carcinomas. In renal carcinomas, hepatocellular carcinomas and squamous cell carcinoma a reduced expression was found in comparison to healthy tissue [23–25]. Furthermore, the tumor suppressor capacity of DUSP9 was confirmed on squamous cell carcinoma and NSCLC tissues in a mouse model. Herein, a lentiviral transfection of these cells with an inducible *DUSP9* transfect led to a reexpression of DUSP9 and was resulting in a repression of tumorigenesis and apoptosis of tumor cells [23–26].

In contrast to the weak expression of the DUSP9 gene in healthy colon tissue, a strong expression could be observed in the early stages of CRC tumorigenesis in a mouse model [27]. But up to now, no studies have been performed about the DUSP9 expression in advanced human CRC tissue. Besides missing information about DUSP9 expression in the later stages of CRC, up to now no studies have been performed about the implication of epigenetic factors, involved in the regulation of DUSP9 expression during the CRC tumorigenesis.

Because of the strong anti-tumorigenesis capacity of the gene product and the key-role as negative regulator of the transcription-activating last MAP kinases, the *DUSP9* gene is a promising target for methylation analysis on advanced CRC samples. This study has been done with 79 CRC samples and the methylation status of the 11 strongest and weakest methylated samples has been compared with their corresponding tumor free tissues. All analysis has been done by quantitative pyrosequencing after bisulfite treatment of DNA. Additionally, the clinicopathological CIMP features postulated by Ogino et al. [11] have been determined for all samples (except MSI status) and integrated into the analysis of the methylation status.[1]

Material and Methods

Clinical Samples and DNA Extraction

All clinical samples involved in this study have been collected over several years by surgical excisions from patients with CRC at the Katharinen Hospital, Stuttgart, Germany and stored as formalin-fixed paraffin-embedded (FFPE) tissue blocks. A total of 79 DNAs from tumor tissues and 22 additional samples from corresponding tumor free samples were enrolled in this study. The tumor classification and the identification of tumor free FFPE blocks or large areas with no tumor cells in the tumor FFPE blocks has been done on standard H&E-stained histological slides. The DNA extraction was done with the KingFisher DNA preparation device (Thermo Fisher). Elution was done in 80–180 μl buffer AE (Qiagen) and the quantity and quality of the DNA was checked photometrically and by test-PCRs for *GAPDH* or *alpha-tubulin*.

BRAF Mutation Screening

The pyrosequencing technique (Qiagen) has been chosen for the detection of activating *BRAF* mutations within the 79 CRC samples. At first, a PCR using the primers and conditions published by Baldus et al. [28] were done for amplifying a

[1] Clinicopathological data are available on request

region of exon 15 of the *BRAF* gene. After successful PCR amplification, a total of 30–50 ng of each PCR product was used for the pyrosequencing reaction. The sequencing reaction worked best with 0.6 μM sequencing primer and has been performed as described in the manufacturers manual (Qiagen), including an oligo- and nucleotide-control. All possible mutations of the codons 599, 600 and 601 were detectable with this assay.

Methylation Analysis

The *DUSP9* methylation was analyzed by quantitative pyrosequencing with the PyroMark Q24 platform (Qiagen). An amount of 2 μg (or the maximum volume: 20 μl) of the DNA extract underwent bisulfite conversion using Epitect Bisulfite Kit (Qiagen). Carrier-RNA was added, as recommended for FFPE tissues by the manufacturer. The chemically treated DNA has been eluted in 20 μl buffer EB.

The *DUSP9* promoter CpG island was determined by 'Methprimer' software (http://www.urogene.org/methprimer/index1.html). This DNA sequence was subsequently used as letter-code in 'PyroMark Assay Design 2.0' software (Qiagen) for creating the *DUSP9* methylation assay, including PCR- and sequencing primer sequences listed in Table 1. The primer binding sites are shown in Fig. 1. The suitability of the primer combinations were tested on clinical samples and 100 % methylated positive control DNA (Qiagen). All listed primers were tested for not generating artificial signals during pyrosequencing (e.g. due to hairpin-loops or self-annealing) as recommended by the manufacturer (Qiagen).

Sometimes the amplification of PCR product 1 failed, due to fragmentized DNA. In that case, the alternative PCR products 1A and 1B were generated, resulting in two PCR products of smaller size, which were still suitable to the sequencing primers listed in Table 1.

In any other case of weak PCR amplification, a re-amplification of these PCR products was performed. Therefore, the PCR products were separated on hydrogel as described by the manufacturer (Elchrom Scientific). The weak bands were picked and transferred into a second PCR reaction, using the same primer combinations as applied in the first PCR round.

PCR amplification was always done in a total volume of 25 μl containing 12.5 μl PyroMark PCR master mix, 2.5 μl Coral Load, 2 μl of each primer [10 μM] and 1–6 μl bisulfite-treated DNA using PyroMark PCR Kit (Qiagen). The PCR cycling conditions were adapted as suggested by the manufacturer for bisulfite treated DNAs with an annealing temperature of 56 °C.

The optimal amount of PCR product for each sequencing primer was determined empirically (see Table 1). The validation of this new established *DUSP9* methylation assay was done on three random picked CRC samples from FFPE tissue. Therefore, the sequencing of each DNA sample was carried out in quadruple including all 11 sequencing primers and the standard deviation has been calculated for each CpG.

Data analysis was performed on pyrograms gained by PyroMark Q24 software (Qiagen) after the sequencing reaction. Each pyrogram was underwent quality controls (e.g. peak height, bisulfite-conversion-controls, expected and received sequence order). Only pyrograms which passed this quality check were used for data evaluation. The methylation strength of each individual CpG ranged from weak (0–33 %), over moderate (34–66 %) to strong (67–100 %), according to the default setting by the manufacturer.

Results

Assessment of the *DUSP9* CpG Island

A CpG island in the promoter region of *DUSP9* with a size of 728 bp was identified in this study. The island is located on chromosome X (NC_000023.10; region 152907596 to 152908324) and includes 83CpG motifs, each representing a potential site for a base modification by methylation.

Validation of the *DUSP9* Methylation Screening Assay

The validation of the newly established *DUSP9* methylation screening assay was done in quadruple for three clinical FFPE samples. The highest standard deviation was detected with 3.5 % at CpG58 for sample 1, for sample 2 at CpG10 with 2.6 % and for sample 3 with 4.7 % at CpG65. The median deviation over all CpGs was 0.78 % (sample 1), 0.8 % (sample 2) and 0.96 % (sample 3). Taken together, the standard deviations obtained for each single CpG motif were quite low in all tested samples, representing a good reproducibility for the sequencing assay in total and an assured determination of the methylation status for each CpG in detail.

DUSP9 Methylation Analysis

The methylation screening by quantitative pyrosequencing was successful for all 79 CRC samples, allowing the exact determination of the methylation status for each CpG motif. The quality of the analysis was good in nearly all cases, only on rare occasions a moderate quality was detected with sequencing primer S8. A summary of the methylation pattern for all tumor samples is shown in Fig. 2.

The individual methylation strength for each CpG motif is accentuated in this figure by a set of different colors. A weak methylation (0–33 %) is represented in blue, a moderate methylation (34–66 %) in green and a strong methylation (67–100 %) in yellow color. The samples were sorted by their methylation strength. Therefore, the strongly and moderately

Table 1 Methylation specific PCR and sequencing primers

Primer	Sequence (5′>3′)	Volume of PCR product
F1	TTTTTGTTTAGGAGGGGTATTGT	
R1*	ACCCAAACCCAACCTAATTCA	
F1A	TGTAAGTTTTTGTTTTTTAGGTTAGTTT	
R1A*	ACCCAAACCCAACCTAATTCA	
F1B	TTTTTGTTTAGGAGGGGTATTGT	
R1B*	ACCCCCACTCATTAACCCCACTAAAAA	
S1	TTTTTAGTGGAGTTAATGAGTG	10 μl PCR product1 of 3–5 ng/μl
S2	AGTTTTTGTTTTTTAGGTTAG	10 μl PCR product1 of 3–5 ng/μl
S3	GGGGTAGAGGTTTGTT	20 μl PCR product1 of 3–5 ng/μl
S4	GGAGGGGTATTGTAG	10–20 μl PCR product1 of 3–5 ng/μl
F2A	GTGTTTTTTTTGGGTAGGG	
R2A*	TACCAAAAAAAACTCCAAATCCC	
S5	TTTTTTTTGGGTAGGGGG	10 μl PCR product2A of 5–10 ng/μl
F2B	GTGTTTTTTTTGGGTAGGG	
R2B*	AAAAACCCCACTACTACAACCTA	
S6	AGGAGGGATTTGGAGTTTTT	10 μl PCR product2B of 5–10 ng/μl
F3A*	GTGGAGTTAGGTTGTAGTAGTGG	
R3A	TCCCCCAAACCCCCCTTACTCCACTCAC	
S7	CTTACTCCACTCACC	10 μl PCR product3A of 5–20 ng/μl
F3B*	AATAGAGTAGAGAGGAGGTGAGTG	
R3B	AAATATACGAAACAACACCGAAT	
S8	CTAAAATATACCTTATCAAAACTTT	10 μl PCR product3B of 5–20 ng/μl
F4	AGGGGTAGTTAGTGTGTGTATTA	
R4*	CCTACACCCCCAAAATCC	
S9	GAGTAGTATAGAATGGGTGT	15 μl PCR product4 of 5–10 ng/μl
S10	GTGTAAGTGGTAGGAGATA	15 μl PCR product4 of 5–10 ng/μl
S11	AGTGTGTGTATTATAGGTAT	10 μl PCR product4 of 5–10 ng/μl

The table shows the different primer names, referring to Fig. 1. The corresponding nucleotide sequences are presented in the second column. Forward and reverse primers are abbreviated as 'F' or 'R'; the sequencing primers as 'S'. Biotinylation is indicated by '*' in the first column. The volume of PCR product per sequencing primer refers to the concentration of the PCR product and is shown in the last column

methylated CpG motifs were counted. The number of strongly methylated CpGs was multiplied by 2 and the moderate by 1 for accentuating the impact of a strong methylation compared to a moderate methylation. Both values were added afterwards and the samples were sorted upon these results from weakly to strongly methylated. The median methylation strength for all samples was 59.48 and is indicated as a red line in Fig. 2.

The 11 samples with the weakest and strongest methylation were identified and the methylation pattern of the corresponding DNA from tumor free tissues was determined and compared to the methylation pattern of the tumor DNA.

In Fig. 3a the methylation status of the 11 weakest tumor DNAs is compared with the corresponding tumor free DNA. The majority of tumor DNAs, 46 % (blue), showed no or only little differences to the normal tissue DNA, while 27 % of the tumor DNA was significantly stronger (red) or weaker (green) methylated.

The situation for the 11 strongest methylated tumor DNAs looks quite different and is presented in Fig. 3b. Only 18 % (blue) of the tumor DNAs showed no or only little differences to the corresponding normal tissue DNA. For the remaining 9 out of 11 cases (82 %; red) a significantly stronger methylation was detectable in the tumor DNA and none of the tested clinical samples showed weaker methylation, compared to the corresponding normal tissue DNA.

Several clinical and molecular CIMP features postulated by Ogino et al. [11] were determined for all tested samples. Interestingly, none of the 11 weakly methylated tumor DNAs could be connected to the CIMP features. But for the 11 strongest methylated tumor DNAs only for 2 out of the 11 samples (18 %, violet) no correlation to any CIMP feature were observed (Fig. 3c). For all the remaining cases (82 %; green) one to several CIMP features were detectable. These 9 samples include 2 *BRAF* mutations, 7 proximal tumors and 7 patients with female sex.

Discussion

The aim of this study was the development of a methylation screening assay for *DUSP9*, which is a strong negative regulator of transcription factor activating kinases (ERK, JNK and

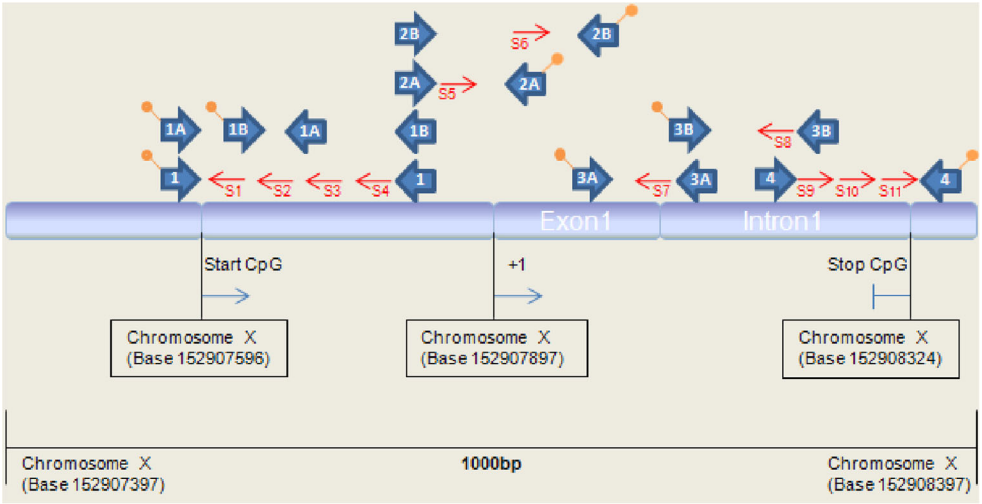

Fig. 1 Schematic overview of the PCR- and sequencing primer binding sites for the *DUSP9* pyrosequencing methylation assay. The promoter region of the *DUSP9* gene, including the first CpG island ranging from Base 152907397 to 152908397 (source: NCBI NC_000023.10 Xq28), is drafted in that figure. The PCR primers are displayed as *blue arrows*, the sequencing primers as *red arrows*. The biotinylated PCR primers are indicated by *orange dots*. As an alternative for the PCR1 primer combination, the PCR1A and PCR1B primer combinations can be applied. Both PCR products are suitable for the appropriate sequencing primers

p38) in the main tumorigenesis pathway for CRC, the MAPK pathway. The investigation was done on 79 clinical CRC samples and some selected corresponding tumor free colon tissues, using pyrosequencing for the determination of the promoter methylation status. The methylation status of the tumor samples were compared to the methylation status of the corresponding tumor free samples and CIMP features were collected, for estimating the usefulness of this gene as a CIMP marker. As far as we know, this is the first study using quantitative pyrosequencing for a *DUSP9* methylation analysis on clinical CRC samples.

Initially, a CpG island of 728 bp could be identified, including 83 CpG motifs. Because the CpG motifs responsible for the transcriptional repression are unknown, the sequencing assay was designed for the investigation of the whole island. The validation of the sequencing assay showed only little deviations, resulting in a high reproducibility of the assay comparable to other pyrosequencing approaches described before [29].

The determination of the methylation status for every sample and over all CpG motifs was successful. The results show large differences between the different CRC samples, ranging

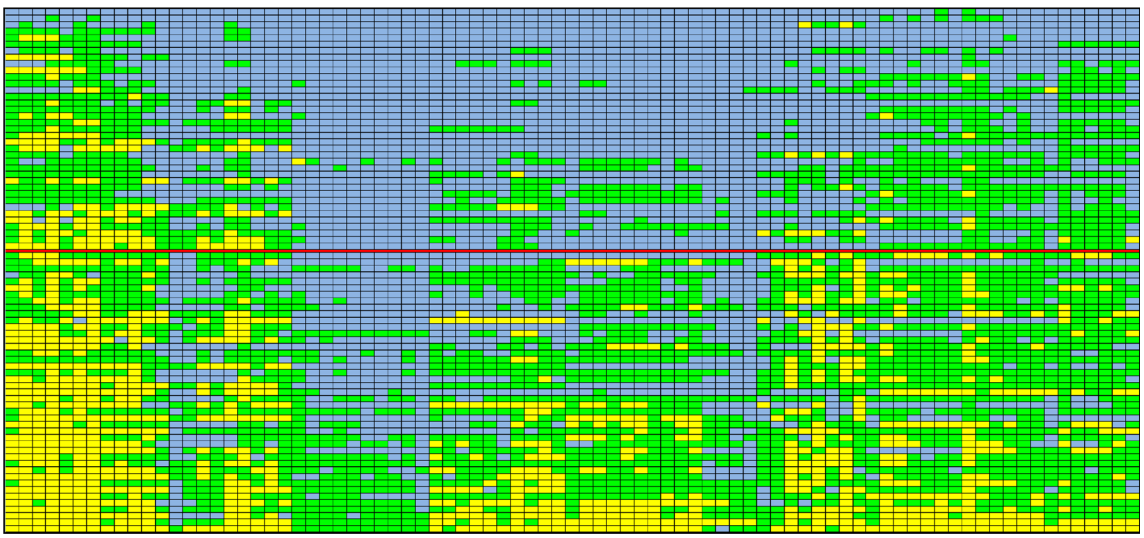

Fig. 2 *DUSP9* Methylation pattern for all CRC samples. The 83 analyzed CpG motifs of the *DUSP9* promoter CpG island are presented on the X-axis, starting at CpG1 and ending with CpG83. The individual methylation strength of each motif is indicated by a color code: weak methylation (0–33 %) *blue*; moderate methylation (34–66 %) *green*; strong methylation (67–100 %) *yellow*. All 79 patient samples can be found on the Y-axis. The samples were sorted by their methylation strength ranging from weak to strong. The last sample on the Y-axis is the three times enzymatically methylated positive control (Qiagen). The median methylation strength (59.48) is indicated by a *red line*

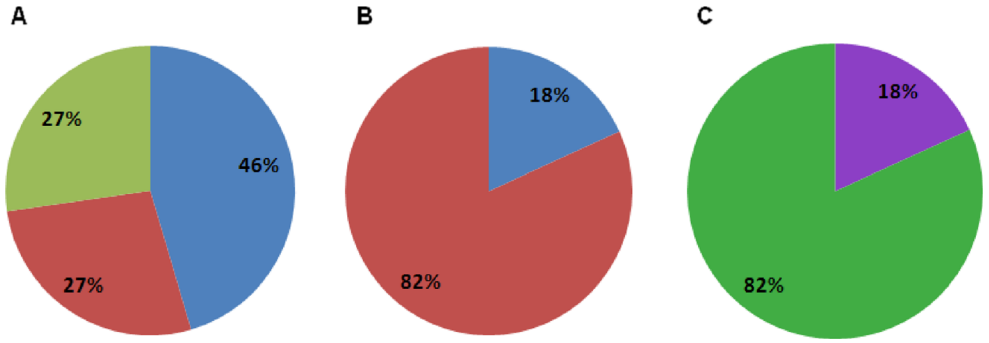

Fig. 3 Comparison of the methylation status between tumor and tumor free tissue DNA extracts and the possible implication of CIMP features. **a** The methylation statuses of the 11 weakest methylated tumor samples were compared with their tumor free counterparts. *Blue*: no or only little differences were found; *green*: methylation strength in the tumor DNA is weaker; *red*: methylation strength in the tumor DNA is stronger. **b** The methylation statuses of the 11 strongest methylated tumor samples were compared with their tumor free counterparts. *Blue*: no or only little differences were found; *red*: methylation strength in the tumor DNA is stronger. **c** CIMP features could be found exclusively for the 11 strongest methylated tumor samples. *Violet*: percentage of CRC samples showing no CIMP features; *red*: percentage of CRC samples showing one or several CIMP features

from nearly completely unmethylated to nearly completely methylated. A differential methylation pattern of the CpG motifs is present in these samples, giving a first hint for a transcriptional regulation of this gene by differential methylation of the CpGs motifs.

Interestingly, we could identify a stretch of CpG motifs (CpG22 to CpG33) where the methylation does not exceed 66 %. The reason for this is unknown. But even the three times enzymatically methylated positive control does not show a higher methylation at these positions, suggesting that this region of DNA may have a limited accessibility for DNA methylases.

The methylation analysis of the 22 corresponding tumor free DNAs from the CRC samples with the weakest and strongest methylation revealed large differences between the two groups. The first group, including the tumor free DNAs from the tumor samples with the weakest methylation, in most cases (46 %) showed no remarkable differences to their tumorgenic counterparts. For the remaining 54 % of samples an elevated (27 %) or reduced (27 %) methylation was detectable. Taken together, the methylation statuses of the corresponding samples from this collection do not appear to be noticeable. Contrary to these findings, the situation for the second group looks quite different: only 18 % of these samples showed a similar methylation status compared to their tumorgenic counterparts, while in all remaining samples (82 %) a significantly stronger methylation of the tumor DNA was found. This could be an indication, that for a subpopulation of patients with CRCs a differential methylation status between tumor and corresponding tumor free DNA is given, which may contribute to the tumorigenesis of CRC and hints towards a differential transcription of *DUSP9* mediated by methylation in a subpopulation of CRC patients.

For further analysis of these samples, several CIMP features published by Ogino et al. [11] were collected for all patients. A first trend can be seen by regarding the samples below and above the median methylation of 59.48 (Fig. 2). The samples below the median include 8 patients of female sex (10.12 %), 4 proximally localized tumors (5.06 %) and no *BRAF* mutation (0 %). A significant increase of these features can be seen for the samples above the median. Herein, a total of 24 samples (30.38 %) are from patients with female sex, 15 samples (18.99 %) have a proximally localized tumor and all 7 *BRAF* mutations (8.86 %) can be found.

Moreover, considering only the 11 samples with the weakest and strongest methylation, no CIMP features were detectable for the 11 weakly methylated tumor samples, while 82 % of the 11 strongly methylated tumor samples showed one to several CIMP features. These findings support the suitability for *DUSP9* as a CIMP marker, but due to the limited number of analyzed samples with weak or strong methylation, again only a trend can be seen.

So far, not much is known about the expression of DUSP9 in human colon tissue. A strong expression can be seen in healthy human placenta tissue, the embryonic liver, adult kidney, insulin-sensitive tissues, migrating muscle cells and testis [21, 30]. In some unpublished studies, an expression in the large colon could be regarded in an expressed sequence tag (EST) study of the 'National Center for Biotechnology Information' (NCBI) by Strausberg (1999). Immunohistochemical investigations by 'The Human Protein Atlas Project' and 'NeXtProt' could verify a weak staining of adult, unstimulated and healthy colon tissue. The weak expression in the healthy colon tissue may be due to the strong physiological effects of this protein and could be sufficient for maintaining the normal cell function in unstimulated, adult colon cells as suggested by Jeffrey et al. [18].

Beside this, a strong expression in the early stages of CRC tumorigenesis could be observed in a mouse model by Sansom et al. [27], but no data exist about the expression in the early stages of human CRC tumorigenesis. It is possible, that the strong expression in the mouse model is due to the

genome-wide demethylation, as described by Feinberg et al. [4], and that a de novo methylation, which may occur during later stages of tumorigenesis, is reducing the expression of DUSP9 in the subpopulation showing the CIMP phenotype.

To further investigate the physiological consequences of the different methylation statuses affecting *DUSP9* in humans, a functional protein detection system is essential and needs to be established. In that context, a clinical follow-up would be interesting for patients with CRC harboring very weak and very strong *DUSP9* promoter methylation. The clinical outcome of these patients may be influenced by their individual *DUSP9* methylation status, due to the strong anti-tumorigenic capacity of the gene product [23, 26]. With a functional protein detection system, it could be further possible to identify single CpG motifs, relevant for a transcriptional silencing, which could reduce the laboratory work for a *DUSP9* methylation screening and making the analysis of larger sample numbers more feasible.

Although some data have been collected in this study, indicating a good suitability for *DUSP9* as a CIMP marker, a higher number of samples need to be tested. Additional, analysis for MSI and a functional protein detection system need to be enrolled in prospective studies. Because of the high physiological effectiveness of the *DUSP9* gene product and it's herein indicated aberrant methylation, these prospective studies promise to be very useful and might reveal further interesting insights.

Acknowledgments This research was supported by a grant of Prof. Dr. med. A. Bosse, head of the Division of Molecular Pathology, Department of Pathology, Katharinen Hospital Stuttgart, Germany

Disclosure of Potential Conflicts of Interest The authors declare that there is no conflict of interest.

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