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TMEM25 is a candidate biomarker methylated and down-regulated in colorectal cancer

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Abstract. The identification of novel genes involved in colorectal cancerogenesis is of high clinical relevance for early diagnosis, applying new therapeutic strategies and monitoring disease recurrence, in order to reduce disease incidence and mortality. Gene silencing through CpG island hypermethylation is a major epigenetic mechanism involved in cancer development. In our study, we aimed to identify and validate novel genes with a tumour specific DNA methylation profile in colorectal cancer.
We performed a whole-genome methylation scan and identified several possible candidate genes that are hypermethylated in tumour in comparison to healthy colon mucosa. Using methylation-specific high-resolution melting analysis in a set consisting of 133 colorectal cancer samples, we were able to confirm an altered CpG site in *TMEM25* in 69.2% (92/133) tumours analysed. Furthermore, the expression of *TMEM25* was found to be significantly lower in tumour tissue. An inverse correlation between hypermethylation of *TMEM25* and *TMEM25* down-regulated expression was observed.
Our results suggest that epigenetic down-regulation of *TMEM25* is cancer-related; we thus suggest that *TMEM25* hypermethylation might play a significant role in altering expression of this gene in colorectal cancer.

Keywords: Colorectal cancer, DNA methylation, array-based methylation profiling, high-resolution melting, *TMEM25* hypermethylation, *TMEM25* down-regulation

1. Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed human malignancies worldwide, with a disease-specific mortality rate close to 33% [1]. It arises as a consequence of the accumulation of genetic alterations (such as gene mutations, gene amplifications etc.) and epigenetic alterations (such as aberrant DNA methylation, chromatin modifications etc.) [2]. The most widely studied epigenetic event is DNA methylation, which occurs in CpG-rich clusters known as CpG islands in regulatory regions of many genes. Almost all housekeeping genes, as well as half of tissue-specific genes, have CpG islands. These regions are not usually methylated but when methylation occurs it can diminish gene expression via transcriptional inactivation [3,4]. Aberrant methylation of a CpG island in promoter gene regions is associated with transcriptional inactivation of tumour-suppressor genes in cancer. The so-called 'CpG island methylator phenotype' or 'CIMP' is observed in almost a third of CRC cases, including the majority of sporadic microsatellite-instable CRCs, which are caused by hypermethylation and causal silencing of the mismatch-repair gene *MLH1*. The CIMP trait has been found to be associated with a variety of clinical, histopathologi-

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cal and epidemiological characteristics [5–7]. Changes in DNA methylation have been reported to occur early in CRC development and are therefore promising as early diagnostic markers [8]. Several epigenetic markers have been discovered in CRC tumours, including *MGMT, CDKN2A, SFRP1* and many others [9]. Another aspect is the prognostic significance of methylation markers. *IGFBP3* and *EVL* have been validated as prognostic markers for CRC and found to be useful in stratifying high risk stage CRC patients who would benefit from adjuvant chemotherapy [10]. The potential reversibility of aberrant DNA methylation by de-methylating therapeutics could make them an excellent target for cancer treatment. Other studies have shown that inhibitors of methyl-transferases induce de-methylation, which leads to increased transcript and protein levels in epigenetically altered cancer cells [11]. Previous reports have shown that hypermethylation of gene promoters may change the expression of cancer related genes in various malignancies, including CRC. The silencing of cancer-related genes is recognized as a key mechanism in tumour initiation and progression [12]. These are all reasons why the epigenetic field is becoming more and more important in cancer research.

In this study, we performed whole-genome methylation profiling of CRC tumours and their corresponding healthy, colon mucosa tissue, using the recently launched HumanMethylation450K array. Applying a large-scale methylation approach, we aimed to search for some novel, epigenetically regulated tumour biomarker involved in CRC cancerogenesis. In order to validate the 450K array methylation results, we used a method with higher specificity, the methylation-specific high-resolution melting method (MS-HRM) independently to confirm the altered methylation status of a specific cancer related gene in a large sample set consisting of 133 CRC tumours. In addition, we wanted to investigate the correlation between hypermethylated DNA and mRNA expression of the candidate gene.

2. Materials and methods

2.1. Patients and tissue samples

Thirty-four fresh tumour samples were collected during surgical colectomy from patients who had been diagnosed with primary colorectal adenocarcinoma. Inclusion criteria were no other cancer than CRC diagnosed, no clinically apparent other colorectal syndromes and no previous radio- or chemotherapy. The patient's age, diagnosis, gender, tumour location, size, nodal infiltration and distant metastasis status were obtained (Table 1). Adjacent, macroscopically normal samples of healthy colon mucosa were taken at least 20 cm away from the tumour site. Normal control tissue samples were collected from the same individual, thus controlling for potential inter-individual variability. In our study we also examined additional 99 DNAs from the set of samples used in our previous study, where the MSI status was analysed [13]. These 99 DNAs were selected according to the appropriate DNA quality and quantity for needs of this study. In Berginc et al. eleven samples out of 99 DNAs were evaluated as being MSI-H. All selected 99 DNA CRC samples were histologically evaluated as being primary adenocarcinomas with different TNM staging and tumour location. The median patient's age at the time of diagnosis was 66 years and 45% (45/99) of them were women. RNA of these samples was not collected.

For negative controls used in MS-HRM, peripheral blood was taken from 20 healthy blood donors. The experimental workflow is shown in Fig. 1. Patients enrolled in the study signed an informed consent form agreeing to participate in the study. The National Medical Ethics Committee of the Republic of Slovenia approved this research (approval reference number: 70/04/09).

2.2. DNA extraction

Tumour and corresponding normal tissue samples were stabilized in RNAlater solution (Ambion) immediately after extraction. Briefly, samples were submerged in RNAlater and incubated at 4°C for at least 24 hours to allow solution penetration throughout the tissue. After incubation, samples were stored at −20°C.

DNA from surgically removed samples was isolated with a QIAamp DNA Mini Kit (Qiagen), according to the manufacturer's recommendations. DNA was eluted in a 200 μL of Buffer AE. DNA used as negative controls was extracted from peripheral blood using a standard salting out procedure [14]. DNA quantity and quality was determined spectrophotometrically by NanoDrop ND-1000 (Thermo Fisher Scientific).

Table 1
Clinical and pathological characteristics of colorectal cancer patients used for confirmatory RT-PCR analysis (In the gender column, M represents male and F female; in Methylation status, M represents methylated and U un-methylated sample; ND stands for not determined)

Patient	Gender	Age at diagnosis	Tumour location	Tumour size (T)	Lymph node status (N)	Distant metastasis (M)	*TMEM25* methylation status
1	F	83	ascending	2	1	0	M
2	F	69	coecum	4	1	0	M
3	M	59	rectum	3	0	0	M
4	M	62	recto-sigmoid	3	0	0	M
5	F	64	rectum	3	0	0	M
6	M	74	sigmoid	3	0	0	M
7	F	78	recto-sigmoid	3	2	ND	M
8	M	78	recto-sigmoid	3	0	0	M
9	M	70	rectum	1	0	0	M
10	M	74	recto-sigmoid	3	0	0	M
11	M	73	ascending	4	0	0	M
12	M	51	ascending	3	0	0	M
13	F	54	sigmoid	3	0	0	M
14	F	54	sigmoid	1	0	0	M
15	F	67	coecum	3	1	0	M
16	M	56	rectum	3	2	0	M
17	M	64	rectum	3	0	0	M
18	M	68	recto-sigmoid	3	2	1	U
19	M	74	ascending	3	0	0	M
20	M	72	rectum	2	ND	1	M
21	M	64	rectum	2	0	0	M
22	M	73	splenic flexure	3	2	0	U
23	F	62	sigmoid	3	0	0	M
24	M	73	rectum	3	1	0	U
25	M	86	transverse	3	0	ND	U
26	M	55	sigmoid	3	1	0	U
27	F	61	coecum	3	0	0	U
28	F	70	rectum	3	2	0	U
29	M	74	rectum	3	1	0	U
30	M	43	ascending	3	0	0	U
31	F	63	sigmoid	3	ND	1	U
32	M	53	sigmoid	3	1	ND	U
33	M	72	rectum	3	1	0	U
34	M	69	recto-sigmoid	3	1	0	U

2.3. Genome-wide methylation profiling

Twelve DNAs (nine tumours and three corresponding healthy colon mucosa tissues) from nine different patients were chosen for genome-wide methylation profiling. DNAs were chosen from set of samples collected for our previous study [13]. The primary criterion of selection was the availability of paired samples with high DNA quantity and quality that met the conditions required for 450K Illumina protocol. Additional criteria were the selection of primary adenocarcinoma samples with different histopathological characteristics regarding tumour size (T status ranging from 1–4), nodal infiltration (4 samples were positive) and site of location (4 from sigmoid colon, 2 from rectum, 1 from splenic colon flexure, 1 from ascending colon and 1 from coecum). DNAs were subsequently bisulphite converted using EZ DNA Methylation Kits (Zymo Research), according to the manufacturer's recommendations for the Illumina Infinium Assay. Genome-wide methylation profiling of all twelve samples was done using HumanMethylation450 BeadChip micro-array technology (Illumina). Paired samples of tumour and adjacent non-tumour tissue were processed on the same chip to avoid chip-to-chip bias. The Illumina Infinium HD Methylation protocol was performed.

2.4. Genome-wide methylation data analysis

Illumina Genome-Studio software was used to generate a ß value for each locus. ß values represent the percentage of methylation of a given cytosine, corresponding to the ratio of the methylated signal over the sum of methylated and un-methylated signals. ß values range continuously from 0 to 1, representing the non-

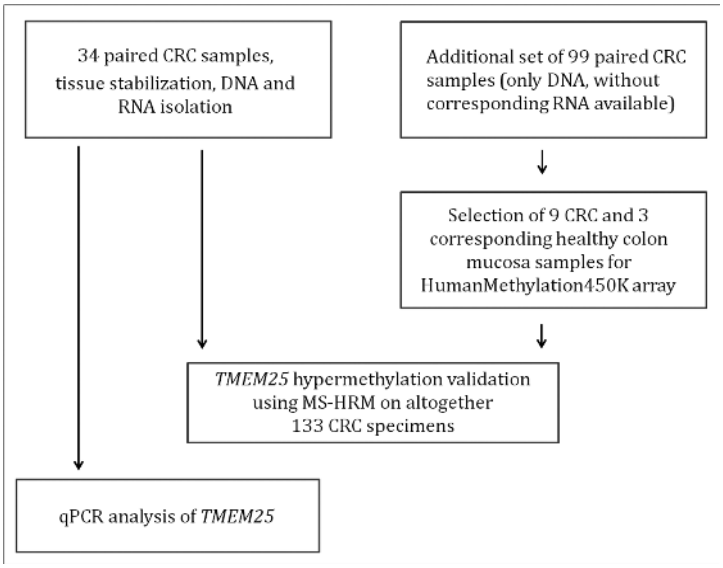

Fig. 1. Schematic representation of the *TMEM25* hypermethylation discovery and verification pipeline. Samples used in the study were from two sets. One consisted of 34 paired CRC samples, which were fresh collected and stabilized. From obtained material the DNA and RNA were isolated. Second sample set was larger and consisted of 99 CRC samples, from which only DNA was isolated. From larger set of CRC samples 12 were chosen for HumanMethylation 450K array. All 133 (34+99) paired CRC samples were used in methylation verification with MS-HRM method, and subsequently only 34 RNA from the smaller group of tumours were available for qPCR analysis.

methylated and methylated sites, respectively. Downstream analyses were conducted using IMA package in R language. A site-level test, with manually performed normalization using the Peak correction method, was performed. Only genes from autosomal chromosomes were included in the analysis. Sites on the sex chromosomes were removed. Sample filter detection was set at 1e-5, meaning that only specific loci whose p value was equal or lower than 1e-5 (0.00001) was considered as significant for further analysis. Sample filtering percentage was set at 0.65. This is the percent of samples with detection p value less than "sample filter detection" for analyzed loci. In other words: specific CpG site was considered significant for further analysis only if its p value was equal or lower than 1e-5 in at least 65% of all samples analyzed. The threshold p value was set at 0.05 after Bonferroni correction applied. SNP sites were filtered out. A pooled t-test, with Bonferroni correction, was performed with the ß difference cut set at 0.14. We compared a group consisting of 9 CRC samples and a group of 3 normal controls. Batch effect was avoided, since all samples were run simultaneously on the same chip.

2.5. *Validation of TMEM25 hyper-methylation by MS-HRM*

For further validation purposes, all 133 (34+99) paired DNA samples were subjected to bisulphite conversion using an EpiTect Kit (Qiagen), following the manufacturer's instructions. Purified DNA was stored at −20°C until use.

Our method of selection for validating array results was MS-HRM. *TMEM25* primers for amplification of the specific region in the 5'UTR region of *TMEM25* were designed using the on-line, freely available software tool Methyl Primer Express Software v1.0 (Applied Biosystems). The primer set sequence used was as stated in Table 2 and was designed to amplify both methylated and un-methylated DNA. Amplicon length was 72 bp and covered the specific CpG site in the 5' UTR region of *TMEM25* that was shown to be hypermethylated in the methylation array. The amplification was performed using an EpiTect HRM PCR Kit (Qiagen), according to the manufacturer's instructions. Briefly, 0.75 μL bisulphite converted DNA, 5 μL HRM EpiTect HRM PCR Master mix (Qiagen), 0.75 μL of each primer and 2,75 dH_2O was added to obtain a total PCR reaction volume of 10 μL. Optimized cycling protocol for HRM analysis on the Rotor-Gene Q (Qiagen) was preformed including: initial denaturation at 95°C for 10 sec, annealing at 59°C for 30 sec, extension at 72°C for 10 sec (using Fluorescence data acquisition on the "Green" channel at this step). Cycle numbers used were 40 and HRM analysis was performed immediately after PCR under the following conditions: 65–95°C with 0.1°C increments every 2 sec. This step

Table 2
Primer sequence and annealing temperature used for MS-HRM

Gene	Primer sequence	Product length	Annealing temperature
TMEM25	F: 5' TGTGTTTTTTTGTATTGTAGTTTGG 3' R: 5' CCAACAAACACATAAACATCCTAC 3'	72 bp	59°C

requires fluorescence data acquisition on the "HRM" channel.

Un-methylated DNA, as negative control, was prepared by mixing DNA isolated from peripheral blood from 20 healthy individuals. Sample DNA used for normalization purposes was prepared by mixing 50% of purchased fully methylated EpiTect Control DNA (Qiagen) in a background of 50% un-methylated bisulphite converted DNA. In each experimental run we included: no template control, purchased fully methylated DNA, prepared un-methylated DNA and a mixture of 50% methylated and 50% un-methylated DNA. All amplifications were performed in duplicate, using Rotor-Gene Q (Qiagen), following the manufacturer's recommendations. Melting normalized and difference graphs were used to analyse the data.

2.6. RNA isolation

Tissue samples preserved in RNAlater (Ambion) were used for RNA extraction with QIAzol Lysis Reagent (Qiagen) and chloroform. After extraction, total RNA was cleaned with a miRNeasy Mini Kit (Qiagen), performing the on-column DNase Digestion step, using RNase-Free DNase Set (Qiagen). The RNA was eluted twice in 30 μL of nuclease free water. The quality of RNA was checked on a 2100 Bioanalyzer (Agilent Technologies) using an RNA 6000 Nano LabChip (Agilent Technologies). The RNA quantity was determined using NanoDrop-1000 (Thermo Fisher Scientific).

2.7. Two-step quantitative RT-PCR

Expression of *TMEM25* in cancer samples relative to their normal control was measured using quantitative real time PCR based on the TaqMan fluorescence methodology (Applied BioSystems). Only samples with RIN (RNA integrity number) above 6.0 were used for RT-PCR analysis. RT-PCR was performed on a smaller set of samples due to RNA availability. RNA was first transcribed to first strand cDNA using TaqMan Reverse Transcription reagents (Applied BioSystems). The reverse transcription reaction was performed in a volume of 50 μL with 1000 ng of total input RNA. The cDNA was five-fold diluted and amplified in 20 μL reactions using an ABI 7900 Real Time PCR System (Applied Biosystems). We evaluated four potential endogenous controls (*GAPDH*, *ß-actin*, *HPRT* and *TBP*) on ten randomly selected CRC samples. NormFinder software [15] mathematically determined the last two to be best performing in our set of samples, so we used *HPRT* and *TBP* endogenous controls for normalization purposes. The primer and probe sequences used have been previously published [16]. All the RT-PCR reactions were performed in triplicate and every run included a no-template control. The relative quantification of mRNA levels (quantity of transcripts of the target in tumours relative to normal tissue) was determined using the $\Delta\Delta$Ct method [17]. The cut-off value for differential expression was set to 1.5 $\Delta\Delta$Ct, meaning the sample was considered to be down-regulated only if it reached the -1.5 $\Delta\Delta$Ct cut-off plateau.

2.8. Data analysis

The paired t test was used to compare differences in the amount of *TMEM25* mRNA between tumour and corresponding normal tissue. The Mann-Whitney U test was used to correlate the methylation status to changed gene expression, tumour location, stage and nodal infiltration. Differences were considered to be significant at $p < 0.05$. All statistical analyses were performed using SPSS version 20 (SPSS Inc., Illinois).

All authors read and approved the final manuscript.

3. Results

3.1. Whole-genome methylation profiling results

The whole-genome DNA methylation profile of 9 tumour samples and 3 corresponding healthy colon mucosa tissues was obtained using micro-array technology. The quality control report showed comparable intensity levels for all internal controls in each sample, thus indicating all samples had similar performance. Global methylation patterns were established within and outside CpG islands. The CpG sites in CpG islands

Table 3
Summary of top 20 ranked most differentially hypermethylated CpG sites between normal tissue and colorectal carcinoma obtained by HumanMethylation 450K array, sorted by highest ß value difference

	TARGET_ID	P-Value	Adjust.Pval	ß-Difference	Mean ß Tumour	Mean ß Normal	Gene	Placement
1	cg08684893	5.12E-10	0.0002	0.8595	0.9129	0.0534	**ZNF225**	TSS1500
2	cg15055817	7.71E-08	0.0271	0.7706	0.8464	0.0758	**DGKG**	5'UTR
3	cg20154403	1.08E-08	0.0038	0.7528	0.9356	0.1828	**ZNF568**	TSS1500
4	cg08188890	5.51E-08	0.0193	0.7520	0.7710	0.0191	**ZNF528**	5'UTR
5	cg15694715	5.47E-08	0.0192	0.7341	0.9359	0.2019	**TMEM25**	5'UTR
6	cg17747005	7.53E-08	0.0265	0.7327	0.8371	0.1044	**PREX2**	TSS1500
7	cg05292954	1.14E-07	0.0402	0.7268	0.8564	0.1295	**ZFP28**	Body
8	cg20702559	5.21E-08	0.0183	0.7255	0.8228	0.0974	**FAM110B**	5'UTR
9	cg01826574	3.35E-09	0.0012	0.7169	0.9344	0.2175	**DMRT1**	Body
10	cg19585597	1.08E-07	0.0379	0.7148	0.8075	0.0927	**LOC134466**	TSS200
11	cg23250910	1.15E-08	0.0041	0.7104	0.7177	0.0073	**ZNF304**	TSS1500
12	cg05038216	1.41E-07	0.0496	0.6945	0.8432	0.1487	**CLIP4**	5'UTR
13	cg09941363	7.57E-08	0.0266	0.6917	0.8041	0.1124	**RNF11**	Body
14	cg14523847	6.34E-08	0.0223	0.6833	0.7918	0.1085	**DPP6**	1stExon
15	cg11312896	1.28E-07	0.0448	0.6779	0.8212	0.1432	**ZSCAN1**	TSS1500
16	cg06141624	1.31E-08	0.0046	0.6769	0.7823	0.1053	**SOX5**	TSS1500
17	cg05163496	7.35E-08	0.0258	0.6743	0.7880	0.1137	**CD8A**	5'UTR
18	cg13971892	2.83E-08	0.0099	0.6738	0.7606	0.0869	**PPP2R2B**	TSS200
19	cg26542254	2.56E-08	0.0090	0.6707	0.8157	0.1450	**MAGI2**	Body
20	cg14442421	1.38E-07	0.0484	0.6686	0.7324	0.0638	**SLC24A2**	TSS1500

were more likely to be hypermethylated compared to the sites outside CpG Islands.

After filtering procedures and performing site-level test (discussed thoroughly in the materials and methods section) we got 113 sites that were significantly ($p < 0.05$, Bonferroni corrected) differently methylated in CRC tumours (9 samples) than in healthy colon tissue (3 samples). 38% (43/113) of CpG sites had the significance level $p < 0.01$ (Bonferroni corrected). The 20/113 top ranked sites with the highest ß difference between CRC and normal control in favour of hypermethylation are shown in Table 3. From the functional genome distribution standpoint, 32% (36/113) altered CpGs were located in proximal promoters (defined as the sum of CpG sites located within 200 bp or 1500 bp upstream of the described transcription start site) and 22% (25/113) in the 5'UTR. Forty-six percent (52/113) of hypermethylated sites corresponded to the 1st exon or gene body location. The mean difference in methylation levels between tumour and adjacent tissue was higher than 0.66 (66%) for all 20 presented CpG sites, in 100% of samples analysed. All non-malignant controls had un-methylated sites in genes presented in Table 3, with a mean ß value no higher than 0.21.

When compared to other published paper on large scale genome methylation profiling [18–20] regarding colorectal carcinoma, our results showed several similarities. Loci belonging to the families of genes such are SLC gene family and ADAMT gene family were hypermethylated in our study as well as in cited references.

3.2. Candidate gene selection and MS-HRM validation

We selected *TMEM25* for further validation due to its hypermethylated status observed in all nine tumour samples run on the HumanMethylation450 Bead Chip (mean ß-difference 0.7341 between tumour and normal tissue samples, $p < 0.001$). Melting profiles of MS-HRM from methylated and un-methylated samples differed by approximately 1.5°C, as shown in Fig. 2, due to the changed nucleotide sequence after bisulphite conversion. In order to deduce the methylation ratio of each sample and define it as methylated or unmethylated, a standard curve with known methylation ratio was used (50% methylated mixed with 50% unmethylated). None of the corresponding healthy controls obtained from the same patient showed a hypermethylating melting curve profile. Based on the melting curve and standard curve appearance, we were able to determine the methylation status for each individual sample. MS-HRM assays detected *TMEM25* methylation in 69.2% (92/133) of tumours analysed. Differential graph of each high-resolution melting profile nor-

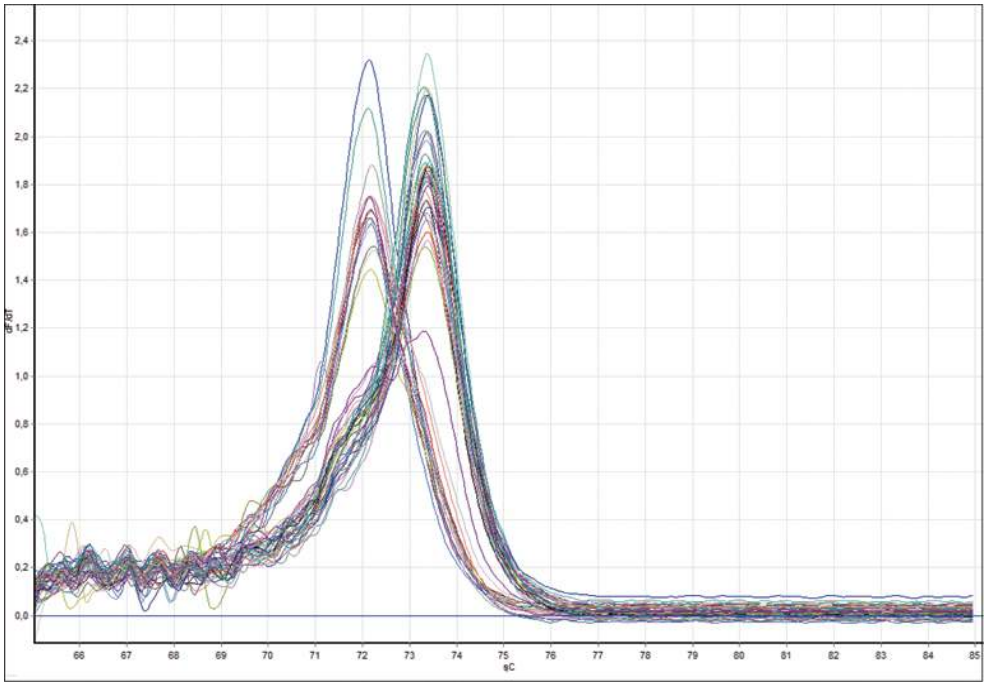

Fig. 2. Methylation-specific high-resolution melting curves of the *TMEM25* gene. Melting curves for each methylated and un-methylated DNA sample. Two different peaks are present for the PCR product derived from un-methylated and methylated sample at the approximately 72°C and 73.5°C, respectively. The sample with the mix of methylated and un-methylated DNA (standard curve) displays two peaks (shown in purple). (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/DMA-120948)

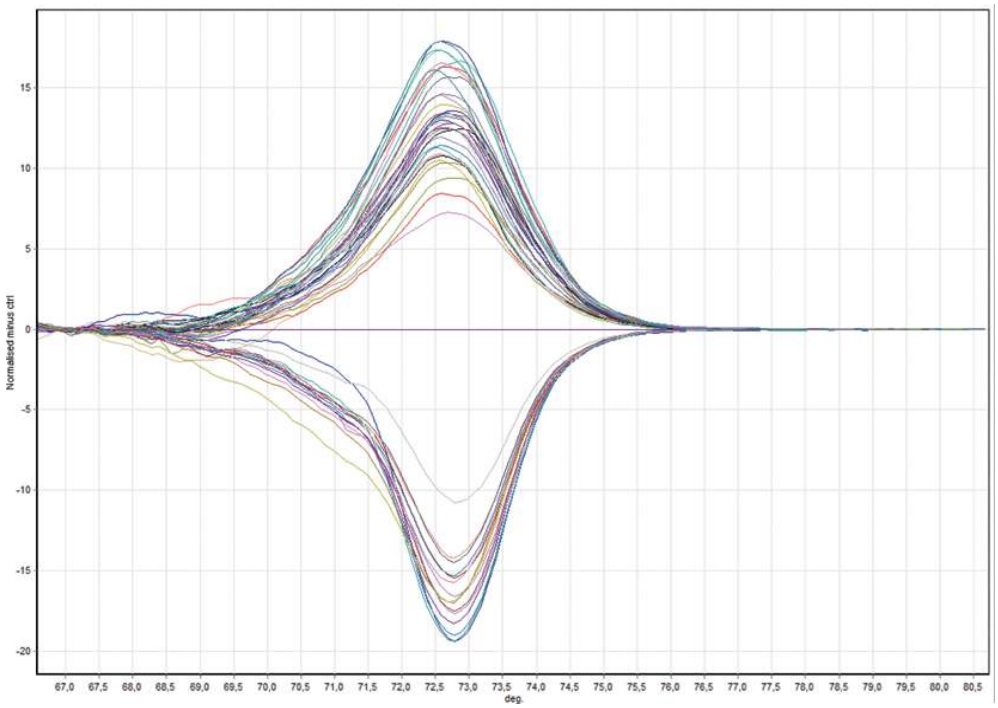

Fig. 3. Differential graph of each high-resolution melting profile normalized against DNA prepared from 50% fully methylated and 50% un-methylated DNA. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/DMA-120948)

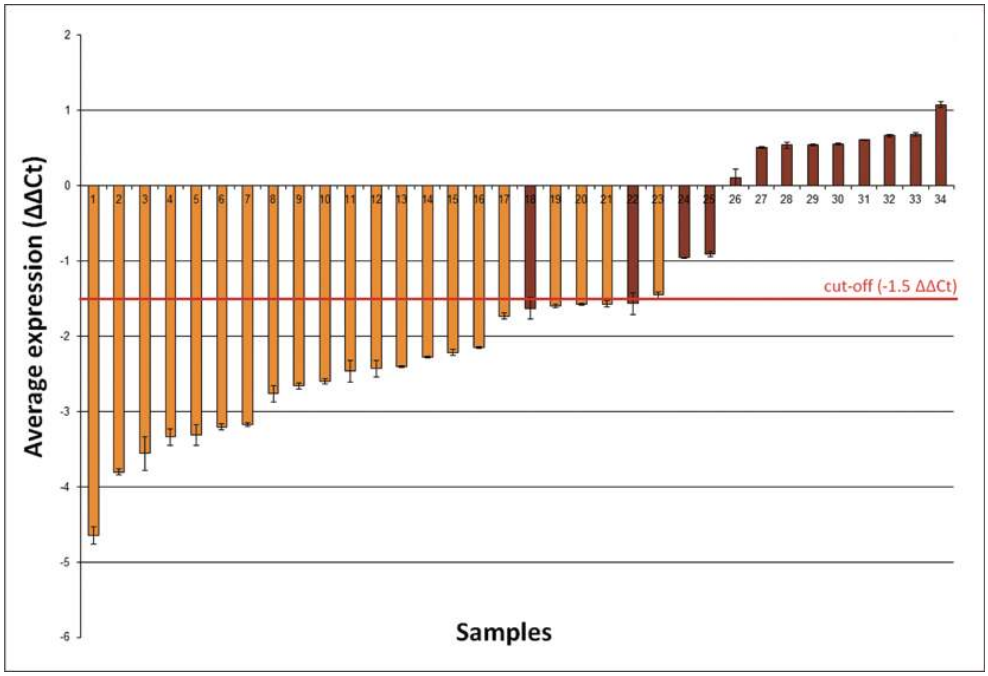

Fig. 4. Differential expression of *TMEM25* in CRC tumours in comparison to corresponding normal tissue ($\Delta\Delta$Ct). Expression is shown in $\Delta\Delta$Ct units, whereby a negative $\Delta\Delta$Ct value represents down-regulation of the gene in CRC in comparison to corresponding normal tissue. The positive bar represents over-expression of the gene in CRC in comparison to corresponding normal tissue. The cut-off is set at -1.5 $\Delta\Delta$Ct (red line). Lighter (orange) colour bars represent tumour samples with hypermethylated *TMEM25* and darker (brown) colour bars represent un-methylated tumour samples. (Colours are visible in the online version of the article; http://dx.doi.org/10.3233/DMA-120948)

malized against a standard curve (Fig. 3) shows how well have methylated samples clustered together on the upper side of graph and all un-methylated samples were clustered together on the opposite side. The normalized melting profiles of the PCR products amplified were consistent between replicates and between different assays. Confirmatory, data obtained for all nine hypermethylated samples from the array platform was in excellent concordance with MS-HRM results, meaning all cancer DNA samples that were shown to be hypermethylated in 450K array were also hypermethylated in MS-HRM validation and all three DNAs from corresponding healthy tissues were not methylated. From the set of 99 CRC samples, eleven were classified as MSI-H, and from those eleven nine were found to have hypermethylated *TMEM25* in our study.

No correlation was found between hypermethylation status and tumour stage, nodal infiltration or tumour location.

3.3. RT-PCR analysis

We additionally performed relative quantification by RT-PCR in order to determine the difference in *TMEM25* mRNA levels between CRC and corresponding healthy tissue. Significant down-regulation of *TMEM25* was detected in 68% of tumours in comparison to corresponding normal tissue. *TMEM25* was clearly down-regulated in 23 out of 34 analysed CRC samples ($p < 0.05$). Figure 4 shows the relative mRNA level of *TMEM25* in each sample analysed. CRC had on average 1.8 times less *TMEM25* than normal tissue, but when considering only down-regulated samples, there was 2.5 times less *TMEM25* mRNA in tumour than in healthy tissue. After quantitative RT-PCR was performed, we observed a strong inverse correlation between hypermethylated *TMEM25* and down-regulation of the *TMEM25* gene for 20/23 cases ($p < 0.001$). Figure 5 shows the correlation between HRM assay results and qPCR presented as the scatter-plot where methylated samples are clustered together and have lower expression level when compared to un-methylated samples.

Two down-regulated samples analysed (numbered 18 and 22, Fig. 4) were not classified as hypermethylated and one hypermethylated (numbered 23, Fig. 4) barely reached the cut-off threshold of -1.5 $\Delta\Delta$Ct. This may be because of the heterogeneity of the sam-

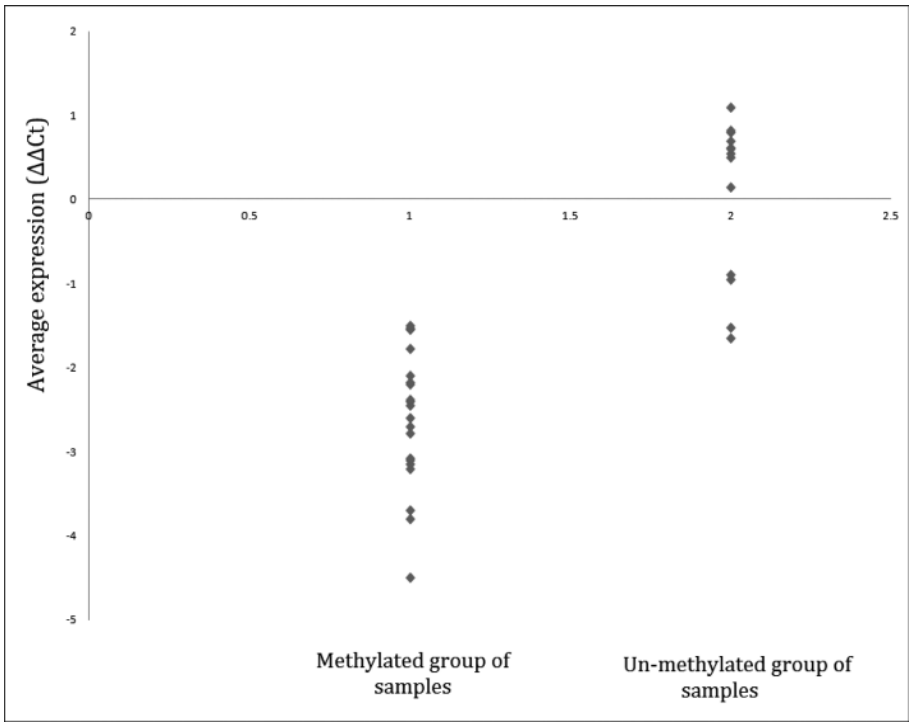

Fig. 5. Scatter-plot showing the correlation between HRM assay results and qPCR. Expression is shown in $\Delta\Delta$Ct units and samples are divided according to their methylation status into two groups (shown on x axis). Hypermethylated samples are clustered together on the left side showing to have lower average expression level when compared to un-methylated samples on the right.

ples taken for RNA and DNA isolation, or there may be some other reason. Those three samples were run in an additional experiment for both MS-HRM and RT-PCR and produced almost identical results.

4. Discussion

In recent years, many scientists and clinicians have come to recognise that epigenetic alterations, such as DNA methylation, are at least as important and disease causing as genetic changes. It is thus very important to focus research on a search for novel epigenetic markers involved in cancer initiation, development, progression and disease recurrence. Since DNA methylation is an epigenetic mechanism considered to be a strong contributor to CRC incidence, we used a large-scale DNA methylation analysis approach to determine the methylation profile of nine tumour samples. The relatively small number of inspected samples chosen is due to the high cost of the array-technology applied. A whole-genome approach is becoming more and more useful in cancer research, as previously reported by many authors [21,22]. The older Infinium Methylation-27 Assay was used in several previously published studies to interrogate methylation status in CRC [18–21]. In our current study, we applied the newly developed Infinium Methylation450K Assay technology, which can simultaneously interrogate over 485,000 cytosine positions distributed throughout the entire genome. This recently launched array enables the reliable measurement of methylation status with single base resolution by quantitative "genotyping" of bisulphite-converted genomic DNA. In parallel, it measures the methylation of protein coding genes, as well as a large number of non-coding RNA genes and imprinted loci [23–25].

Raw data from a 450K array given as the output file from GenomeStudio software was imported into R and analysed using the IMA package. IMA was designed to automate the pipeline for methylation analysis on a 450K DNA methylation micro-array [26]. The Peak-based correction method was used for manual normalization of the data, as suggested by a recently published paper. This method rescales the output data and significantly improves the quality of the obtained results. Due to the divergence between the ß-values retrieved from the two type Infinium assays in 450K, one can improve the obtained output results by using this new correction technique [27].

We identified 113 CpG sites that were hypermethylated in tumour DNA in comparison to normal DNA derived from healthy colon mucosa. Due to the manageable number of differentially methylated sites, we crosschecked their methylation status and involvement in tumourigenesis in already published data in PubMed. Some CpG sites from Table 3 belong to genes that have already been shown to have a cancer related hypermethylated promoter, such as *DMRT1* in gastric carcinoma. This gene was aberrantly methylated in almost half of gastric cancers inspected, but showed no correlation with survival [28]. The *PPP2R2B* gene was found to be hypermethylated in colorectal carcinoma and this mechanism was connected to therapeutic resistance [29]. Recently discovered hypermethylated *LOC134466* in ovarian cancer was proposed as a potential biomarker due to the correlation observed between methylation specific low- and high-grade ovarian cancers [30]. Because of the highly methylated profile of the aforementioned genes obtained in our study, they can all also serve as candidate genes for further research in colorectal carcinoma.

In addition, some of the genes listed in Table 3 have never before been reported as being hypermethylated, such as the *SOX5* gene, which encodes a member of the SOX family of transcription factors involved in the determination of cell fate. The encoded protein may act as a transcriptional regulator after forming a protein complex with other proteins. This novel candidate gene could serve as a putative gene to be further analysed in view of its cancer related, CRC specific methylation profile observed in our study.

From our list of top 20 ranked hypermethylated CpG sites, we chose transmembrane 25 gene (*TMEM25*) for further validation. Since only recently has there been notable research of *TMEM25*, its mechanism of activity and impact on cancerogenesis still remains unclear. It was initially identified *in silico* and characterised as a member of the immunoglobulin super-family, which is implicated in immune response, growth factor signalling and cell adhesion. It was defined to encode transmembrane-type as well as secreted-type protein. *TMEM25* has been found expressed in brain, neuroblastoma, brain tumour and gastric cancer but, as far as we know, never before investigated in CRC. *TMEM25* has been characterized as a target of pharmacogenomics in the field of oncology and regenerative medicine [31]. A previous study investigated the relevance of *TMEM25* as a putative marker in breast cancer. This gene was found to be under-expressed in half of cancers inspected. A strong correlation between expressed *TMEM25* mRNA and lower-grade tumours was also observed and *TMEM25* was thus proposed as an independent, prognostic factor for relapse-free survival. Furthermore, for patients in their study who received adjuvant chemotherapy, significantly longer survival times were achieved if tumours expressed *TMEM25*. They suggested this gene as a useful member of a panel of favourable prognostic and predictive markers, due to its down-regulation in breast cancer tissue. The mechanism causing the down-regulation was not further investigated [16]. Because of the major, cancer related methylation pattern observed in the 5'UTR region of *TMEM25* in our micro-array study, we decided further to investigate this gene specifically.

A problem that arises when using high throughput technologies for marker identification is the lack of sufficient specificity [32]. It is thus necessary to validate array data by some confirmatory method with higher specificity. We used the MS-HRM method for this purpose, which enables detection of a methylated template in an un-methylated background with high sensitivity [33]. This is a cost- and labour-efficient, sensitive screening in-tube method, which applies high resolution melting technology to discriminate methylated from un-methylated template DNA [34]. The method requires DNA bisulphite conversion, during which the target DNA is incubated with sodium bisulphite. The reaction causes the un-methylated cytosines to deaminate into uraciles, while methylated cytosines remain unchanged. MS-HRM can discriminate the melting profiles due to the difference in melting temperatures of the bisulphite treated DNA sequence template, according to the cytosine content. The normalized, output graphs produced from MS-HRM analysis show the degree of reduction in fluorescence over a temperature range. This method is a sensitive, rapid, high-throughput and reproducible technique widely used both in research and diagnostic applications [33, 35,36]. It is important to highlight here the importance of the PCR product length. We designed primers to amplify a relatively short template fragment, in order to make a single nucleotide change more distinguishable because the higher GC content of methylated DNA makes the PCR product more resistant to melting. Even a single base substitution can change the melting temperature of a PCR product by up to 1°C [37]. Using this method, we verified that *TMEM25* has a hypermethylated CpG site in the 5'UTR region in a high proportion of CRC tumour samples. The method was found to be accurate and highly specific, as can be clearly seen in the obtained melting profile, as well as in the

high resolution curves presented in Fig. 2. The high specificity achieved by this approach when analysing the specific *TMEM25* CpG site could be of great importance in further research; for example to investigate whether the method is sensitive enough to determine aberrant methylation of *TMEM25* in tumour-derived, methylated DNA circulating in blood serum. The tumour samples used in our study were human primary tumours, since according to recent studies there may be a significant difference in DNA methylation profiles between cancer cell lines and original tumour tissue [38].

Although the correlative evidence between hypermethylation and down-regulation of gene expression has been accumulating and the importance of CpG island hypermethylation in tumourigenesis has been increasingly recognized, there is a dispute as to whether CpG island hypermethylation is a cause or a secondary event in gene silencing. Nevertheless, DNA methylation may still be an important regulator of transcriptional repression due to the fact that cancer cell lines with a hypermethylated CpG island and down-regulated particular genes can re-induce their lost expression if treated with specific inhibitors of DNA methyl-transferases [8].

Our results support previous findings that a methylated, CpG rich region can lead to transcriptional inactivation of an altered gene. Methylation, as a down-regulation causing mechanism, has been well characterized in various papers published to date [8,10,19, 20,28–30,38,39]. We were able to provide solid evidence of a significant correlation between *TMEM25* hypermethylation and general *TMEM25* mRNA down-regulation. These findings need to be substantiated with additional studies, such as with *in vitro* confirmation using 5-aza-2'-deoxycytidine, to see what effect a de-methylating agent has in restoring *TMEM25* expression.

To date, there have not yet been any publications evaluating the importance of hypermethylation and changed expression level of the *TMEM25* gene in CRC. We therefore propose hypermethylation as a cause of *TMEM25* down-regulated expression. These findings might help in explaining the role of this gene in cancerogenesis and as well elucidating its possible function as a tumour biomarker in colorectal cancer.

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