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RESEARCH

Bisulfite-Based DNA Methylation Analysis from Recent and Archived Formalin-Fixed, Paraffin Embedded Colorectal Tissue Samples

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Abstract We aimed to test the applicability of formalin-fixed and paraffin-embedded (FFPE) tissue samples for gene specific DNA methylation analysis after using two commercially available DNA isolation kits. Genomic DNA was isolated from 5 colorectal adenocarcinomas and 5 normal adjacent tissues from "recent", collected within 6 months, and "archived", collected more than 5 years ago, FFPE tissues using either High Pure FFPET DNA Isolation kit or QIAamp DNA FFPE Tissue kit. DNA methylation analysis of *MAL*, *SFRP1* and *SFRP2* genes, known to be hypermethylated in CRC, was performed using methylation-sensitive high resolution melting (MS-HRM) analysis and sequencing. QIAamp (Q) method resulted in slightly higher recovery in archived (HP: 1.22±3.18μg DNA; Q: 3.00±4.04μg DNA) and significantly ($p<0.05$) higher recovery in recent samples compared to High Pure method (HP) (HP: 4.10±2.91μg DNA; Q: 11.51±7.50μg DNA). Both OD260/280 and OD260/230 ratios were lower, but still high in the High Pure isolated archived and recent samples compared to those isolated with QIAamp. Identical DNA methylation patterns were detected for all 3 genes tested by MS-HRM with both isolation kits in the recent group. However, despite of higher DNA recovery in QIAamp slightly more reproducible methylation results were obtained from High Pure isolated archived samples. Sequencing confirmed DNA hypermethylation in CRCs. In conclusion, reproducible DNA methylation patterns were obtained from recent samples using both isolation kits. However, long term storage may affect the reliability of the results leading to moderate differences between the efficiency of isolation kits.

Keywords DNA methylation · Colorectal cancer · Genomic DNA isolation · Methylation-sensitive high-resolution melting analysis · Pyrosequencing

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Introduction

The majority of surgically removed tissue samples are routinely processed to formalin-fixed, paraffin-embedded (FFPE) specimens and used for pathological and molecular diagnosis. This tissue specimen preservation is cost-effective compared to snap-frozen tissue collection and several established molecular biology tests are already optimized for FFPE starting material [1–4]. However, the presence of cross-linked and highly degraded nucleic acids – the characteristics of these samples that are influenced by variable storage conditions, e.g., temperature, light and oxygen exposure adversely affecting nucleic acid integrity [5] - render the molecular analysis of these samples challenging [6, 7]. Furthermore, in FFPE samples older than 5 years, the abovementioned disadvantages

can be more pronounced due to the longer storage time, which can be a limitation to retrospective studies. Colorectal cancer (CRC) is the third most common malignancy worldwide with approximately 1.2 million new cases worldwide and 600.000 deaths annually [8]. It is well known that this heterogeneous disease can evolve through at least four molecular pathways [9]. On the basis of known genetic alterations the complex process of carcinogenesis still can not be fully explained, thus analysis of disease-associated epigenetic alterations can provide further insight to colorectal tumorigenesis. DNA methylation is one of the most studied epigenetic regulatory mechanisms. Hypermethylation of numerous genes leads to decreased expression that can contribute to CRC formation, including secreted frizzled-related protein 1 (*SFRP1*) [10], secreted frizzled-related protein 2 (*SFRP2*) [11–13] and T-cell differentiation protein (*MAL*) [14], known to be hypermethylated in benign and malignant colorectal tumors when compared to healthy colonic tissue controls. Of the molecular biology methods used for DNA methylation analysis, methylation-sensitive high resolution melting (MS-HRM) analysis is a cost-effective and widely used technique [15]. For the comprehensive screening of DNA methylation alterations of genes potentially involved in colorectal cancer development and progression we aimed to study the reliability of DNA isolation techniques from FFPE tissues with different storage time.

Here we tested two genomic DNA isolation methods using archived (>5 years) and recent (<6 months) FFPE tissue samples and two commercially available kits. To validate the downstream applicability of isolated DNA samples, DNA methylation analysis for *SFRP1*, *SFRP2* and *MAL* genes was performed using MS-HRM analysis and GS Junior sequencing.

Materials and Methods

Patients and Sample Collection

Surgically removed colorectal cancer (CRC, UICC stage II-III) (n=10) and corresponding normal adjacent tissue (NAT) (n=10) specimens were collected and fixed in buffered formalin and embedded in paraffin. Two groups of FFPE blocks were selected on the basis of storage time: archived samples older than 5 years (5 CRC, 5 NAT) and recent samples collected within 6 months (5 CRC, 5 NAT). The study was approved by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics (ETT TUKEB 23970/2011) and written informed consent was provided by all patients.

Genomic DNA Isolation

Serial sections (10 μm) were cut from FFPE blocks and dewaxing was performed with incubation in xylene for 2× 10 min and in absolute ethanol for 2×10 min. DNA was isolated from four consecutive sections from each sample using High Pure FFPET DNA Isolation Kit (Product ID: 06650767001, Roche Applied Science, Penzberg, Germany) and from four sections using QIAamp DNA FFPE Tissue Kit (Product ID: 56404, Qiagen GmbH, Hilden, Germany) according to the manufacturers' instructions, with the following modifications. Proteinase K digestion at 56°C was extended to 2 h with shaking at 600 rpm for archived FFPE samples in the Roche protocol and for both archived and recent samples in the Qiagen protocol. Elution volumes were 100 μl for recent samples and 50 μl for archived samples.

Quantitative and Qualitative Analysis of the Isolated DNA Samples

Concentrations and purity ratios of isolated nucleic acid samples were measured with NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Fluorometric analysis was performed in order to measure DNA and RNA content in the isolates using Qubit 1.0 fluorometer (Invitrogen, Carlsbad, USA) using Qubit dsDNA HS Assay or RNA HS Assay, respectively.

Bisulfite-Specific PCR (BS-PCR) and Methylation-Sensitive High-Resolution Melting Analysis (MS-HRM)

Bisulfite conversion was performed using EZ DNA Methylation™ Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. Input DNA was 1 μg from both recent and archived FFPE samples or 100 ng DNA from archived samples with low concentration based on OD260 measurements. Concentration of bisulfite converted samples was estimated by 'ssDNA' NanoDrop measurements. Bisulfite-specific PCR primers were designed with PyroMark Assay Designer software (Qiagen) to amplify bisulfite-converted DNA (bcDNA) without discriminating between methylated and non-methylated sequences (Table 1). BS-PCR reactions were performed using AmpliTaq Gold 360 Master Mix (Life Technologies, Carlsbad, USA), LightCycler® 480 ResoLight Dye (Roche Applied Science), primers at 0.2 μM final concentrations, 1.5 mM final concentration of $MgCl_2$ for *SFRP2* and 2 mM $MgCl_2$ for *MAL* and *SFRP1* assays, 20–40 ng bcDNA sample in 15 μl final volume. Real-time PCR amplification was carried out with the following thermocycling conditions on the LightCycler® 480 System: 95°C for 10 min, then 95°C for 30 s, 60°C with 0,4°C decreasement/cycle for 30 s, 72°C for 30 s for 10 touchdown

Table 1 Analyzed markers, primer sequences, genomic location of amplicons and amplicon lengths

Gene Symbol	Primer sequence/forward (F) and reverse (R)/	Amplicon location	Amplicon length
MAL	F: TGGTGAAGATAGAGAAGTTATTGGGTAGG R: AAAACCCCCAAACCACTAAACTC	Chr 2: 95691438–95691598	160 bp
SFRP1	F: GGAAAGAGATAAGGGGAGAAAAAGAA R: ATTTCATAAATTTACAAATATAATCCAAACTCC	Chr 8: 41167194–41167333	139 bp
SFRP2	F: GGTTAAGATAGGTTTAATTGATTATTGGGGAATAG R: TAAACACCCAATATCCCATCCCT	Chr 4: 154712515–154712634	119 bp

cycles, followed by amplification at 95°C for 30 s, 53°C for 30 s, and 72°C for 30 s in 40 cycles. HRM analysis began with denaturation at 95°C for 1 min, cool down to 40°C, and hold for 1 min, then continuous warm up to 95°C with 20 acquisition/°C rate during melting curve fluorescence acquisition. A derivative curve-based line diagram of dF/dT called melting peak diagram is a common way to present results of HRM analyses. As melting peak diagrams are difficult to interpret for many samples, data were also visualized on heat maps representing height values of melting peak curves on a color scale (red: high, green: low intensity of fluorescent signal) for each sample (columns) at different temperatures (rows) [16]. In order to calibrate our MS-HRM assays, in vitro fully methylated and unmethylated DNA standards (EpiTect® Control DNA, Qiagen) were mixed in different ratios and used as methylation control samples (0%, 10%, 25%, 50%, 75%, 100%) for MS-HRM*)*. Average methylation levels of all analyzed samples were estimated by two experts independently by visually comparing melting peak curves with those of standard samples.

GS Junior Sequencing

BS-PCR products from two normal and two recent FFPE CRC samples isolated with High Pure method (N2, N4, T2, T4) were analyzed by GS Junior sequencing according to the manufacturer's protocols (454 Sequencing System Guideleines for Amplicon Experimental Desing and Amplicon Library Preparation Method Manuals). Fragment End Repair was performed using GS FLX Titanium Rapid Library Preparation Kit. Rapid Library Molecular Identifier (RL MID) Adaptor Ligation was carried out using GS FLX Titanium Rapid Library Preparation Kit. Equimolar mixing of the libraries was performed with different MID adaptors for the individual samples. EmPCR amplification was performed using the Lib-L emPCR Kit (Roche) with 0.5 DNA molecule:bead ratio. Bead enrichment and sequencing were performed using GS Junior Titanium Sequencing Kit according to the Sequencing Method Manual. The reads were matched to template sequences using the Smith-Waterman algorithm with Gotoh's improvement [17, 18] as implemented in the JAligner software package. As due to the characteristic of 454 sequencing technology bisulfite-sequencing PCR products with homopolymer stretches can result in sequencing errors [19], the number of nucleotides in homopolymers was often miscounted, resulting in gaps or insertions in the sequenced reads. For statistical analysis reads with at least 80% of the maximum alignment score were retained, then the actual nucleotides at the potential methylation sites were summarized.

Comparison of MS-HRM Results With TCGA Data

DNA methylation alterations of *MAL*, *SFRP1* and *SFRP2* were also analyzed on dataset downloaded from The Cancer Genome Atlas (TCGA) [20] as a confirmatory analysis on independent samples. HumanMethylation450k BeadChip data were downloaded from the website and beta values of *cg22403344 probe (MAL), cg04255616 probe (SFRP1) and cg25185173 probe (SFRP2)* were retrieved belonging to matched healthy colonic and colorectal tumor samples available (n=38). TCGA data and our results were visualized together on box plots.

Results

Quantity and Quality of the Isolated Genomic DNA

Nucleic Acid Recovery Based on Spectrophotometry (OD260) Measurements

In both archived and recent FFPE groups, normal samples had lower amounts of isolated nucleic acid than tumor samples, although it was only due to the relatively smaller tissue area processed (mean area: 9.6×10^7 μm^2 for normal samples and 3.5×10^8 μm^2 for tumors). Regarding the yield of nucleic acid isolation, the two methods performed similarly from archived samples (median yield±SD; High Pure method: 1.22±3.18 μg DNA; QIAamp method: 3.00 ±4.04 μg DNA), while the QIAamp method yielded significantly higher amounts of nucleic acid from recent samples (median yield±SD; High Pure method: 4.10± 2.91 μg DNA; QIAamp method: 11.51±7.50 μg DNA) (Fig. 1). Besides the significantly higher OD260/280 ratios in eluates from the QIAamp method, OD260/230

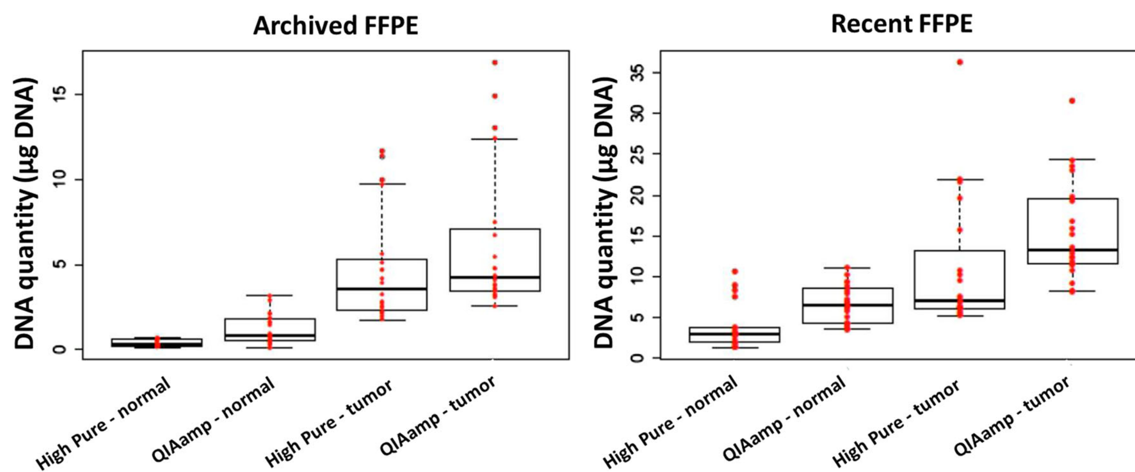


Fig 1 Nucleic acid recovery from archived and recent FFPE samples based on spectrophotometry (OD260) measurements

ratios were also significantly higher than those from the High Pure method from both archived and recent DNA samples (data not shown).

Nucleic Acid Recovery Based on Fluorometric Measurements

According to fluorometric measurements, QIAamp method recovered more DNA from recent samples than did the High Pure method, but not from archived samples. Importantly, RNA content was relatively high in QIAamp samples, while minimal amount of RNA could be detected in the High Pure samples.

DNA Methylation Analysis

Bisulfite Converted DNA Yield After Bisulfite Treatment

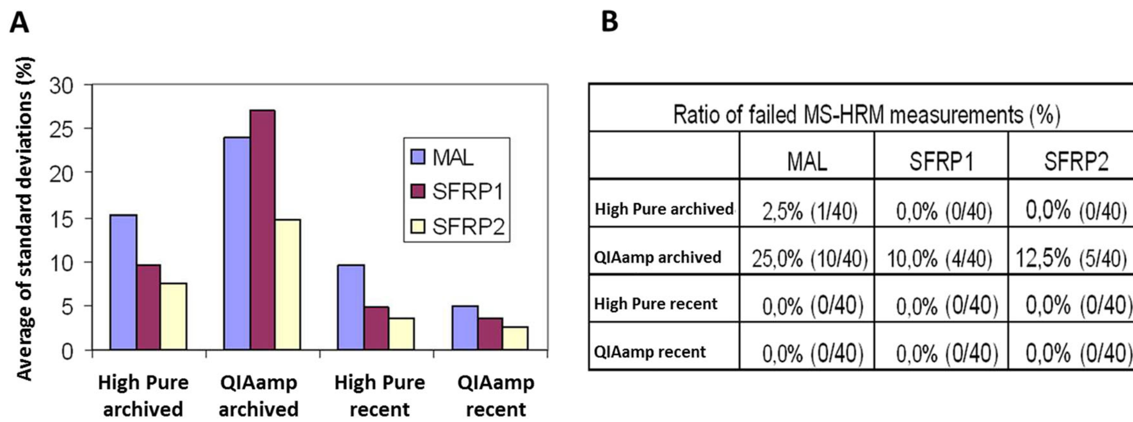
High Pure isolated samples were found to have significantly higher amount of output DNA after bisulfite conversion compared to the QIAamp method in the majority of tumor samples. It should be noted that the amount of input DNA for bisulfite conversion was likely lower in case of QIAamp samples. The input DNA amount was calculated on the basis of OD260 spectrophotometry measurements, that could be influenced and overestimated by the possible high RNA content in case of QIAamp isolated samples. According to the melting peak analysis, all three primer pairs (*MAL*, *SFRP1, SFRP2)* were found to reliably estimate methylation percentages of the standard samples.

DNA Methylation Levels of the Selected Genes

MS-HRM reactions from all recent samples were successful and could be evaluated, while in case of archived samples the number of reactions with no reliable melting curve were significantly higher in QIAamp samples, than those of High Pure ones suggesting the better reproducibility of MS-HRM after High Pure DNA isolation (Fig. 2). The average variance of estimated methylation levels of the four technical parallels was higher in QIAamp samples, and this difference was significant in case of *SFRP1* primer pairs (Fig. 2b). Regarding MS-HRM confidence, the most typical difference between the archived and recent FFPE samples was the frequent overestimation of methylation levels in archived samples. This was particularly confusing in case of normal samples, where unmethylated results were expected on the basis of literature data and TCGA data (Fig. 5). Matched tumor and normal samples were also compared, providing information on methylation changes in individual cases. In this regard the same results were obtained from recent samples using either of the isolation methods (Fig. 3a). Only two marginal differences were found in case of sample T5, where higher degree of hypermethylation was established for *MAL* after QIAamp isolation and the same for *SFRP1* after High Pure isolation. Despite of the harmony between MS-HRM results of QIAamp and High Pure isolated recent samples, substantial discrepancies were found in case of archived ones. For example, contradictory results were found for all three examined genes in sample T3, where hyper- or hypomethylation was observed with one method, while no change in methylation was detected in samples isolated with the other method (Fig. 3b). The ratio of archived samples having concordant MS-HRM results with both DNA isolation methods was 3/5 for *MAL*, 3/5 for *SFRP1*, and 4/5 for *SFRP2.*

GS Junior Sequencing

According to the results of sequencing experiments, promoters of *MAL*, *SFRP1* and *SFRP2* genes were hypermethylated in cancer samples compared to normal samples (Fig. 4)*,* which is in accordance with MS-HRM results and also with literature data.



Ratio of failed MS-HRM measurements (%)			
	MAL	SFRP1	SFRP2
High Pure archived	2,5% (1/40)	0,0% (0/40)	0,0% (0/40)
QIAamp archived	25,0% (10/40)	10,0% (4/40)	12,5% (5/40)
High Pure recent	0,0% (0/40)	0,0% (0/40)	0,0% (0/40)
QIAamp recent	0,0% (0/40)	0,0% (0/40)	0,0% (0/40)

Fig 2 The average variance of estimated promoter methylation levels in four technical parallels (**a**) and the ratios of failed MS-HRM measurements using *MAL, SFRP1* and *SFRP2* specific BS-PCR primer pairs in recent and archived FFPE samples (**b**)

DNA Methylation Alterations on the Basis of TCGA Data

On the basis of beta values of the probes belonging to the three analyzed genes from an independent TCGA dataset significant DNA hypermethylation was observed in all three analyzed gene promoters in colorectal tumors compared to their matched normal tissue samples ($p<0.05$). Similarly, DNA hypermethylation could be detected in our study although on different extent in the archived and recent FFPE samples after the different isolation methods (Fig. 5).

Discussion

Analysis of epigenetic gene regulation processes such as DNA methylation can facilitate the understanding of

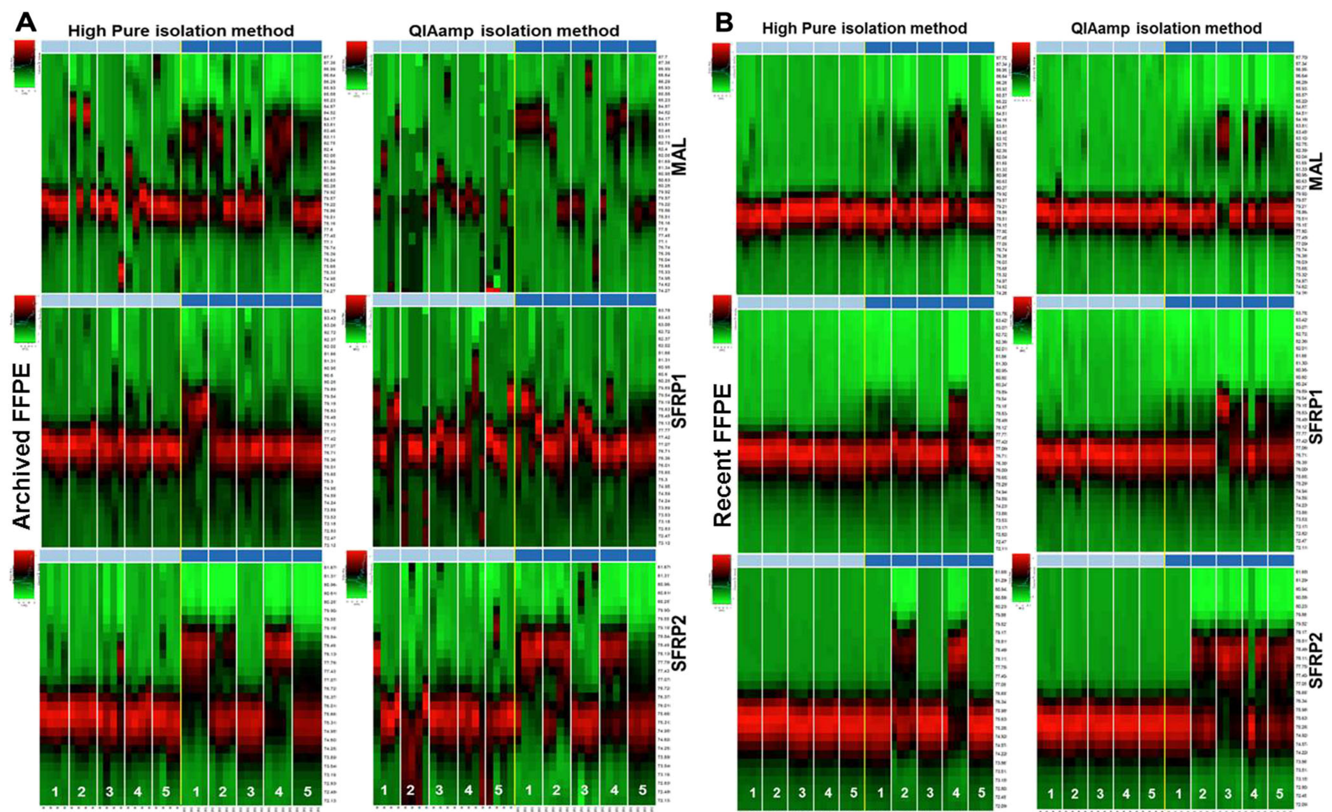


Fig 3 Heat maps of MS-HRM analyses of *MAL, SFRP1* and *SFRP2* promoter methylation levels in archived FFPE (**a**) and recent FFPE (**b**) samples. Heat maps represent intensities in fluorescent signals corresponding to melting peaks plotted against the applied temperature range. Intensity values on the color scale were as follows (red: high intensity, black: intermediate intensity, green: low intensity) for each sample (columns) at different temperatures (rows)

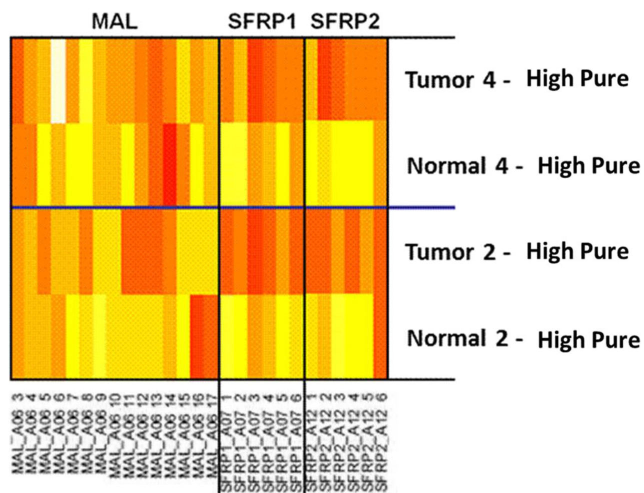


Fig 4 Results of amplicon sequencing performed with GS Junior next generation sequencer. The heatmap shows the methylation status of CpG sites (columns) covered by the amplicons of *MAL, SFRP1* and *SFRP2* specific BS-PCR assays. Color scale: white: unmethylated, red: methylated

colorectal cancer carcinogenesis. The results of the increasing number of such examinations largely depend on the accuracy and reliability of the applied methods. In the present comparative study archived and recent FFPE colon tissue samples were isolated with two different commercially available DNA isolation kits and used for DNA methylation analysis of three colorectal cancer associated genes (*SFRP1, SFRP2* and *MAL*). The QIAamp method resulted in a slightly higher nucleic acid yield from archived and a significantly higher nucleic acid output from recent FFPE samples compared to the High Pure method. Both methods yielded similar DNA content from archived FFPE samples, while more DNA could be obtained from recent FFPE samples when using the QIAamp method. Besides genomic DNA, a remarkable quantity of RNA was present in the isolated samples using either of the two methods. In parallel, a higher RNA content was found in QIAamp isolates from both archived and recent FFPE samples. As it could also be expected from literature data [5], the difference in RNA quantity compared to the High Pure method was lower in archived FFPE sample group probably caused by the extended RNA degradation due to the longer storage time.

The OD260/280 ratio was found to be higher in QIAamp samples, that can be caused by the higher RNA quantity in QIAamp isolated samples, as RNA has a higher 260/280 ratio due to its uracil content and indicates that the eluates are free of protein contamination.

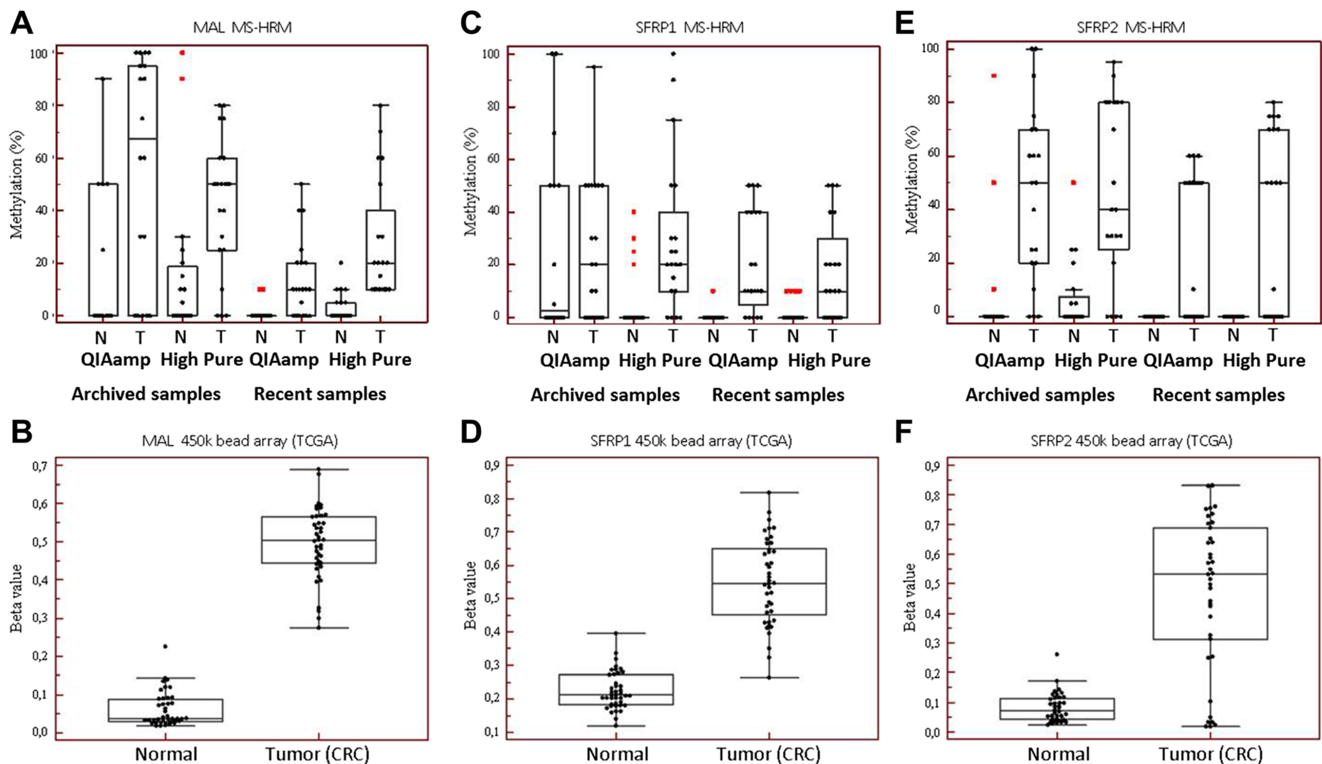


Fig 5 Comparison of the MS-HRM results of FFPE samples with methylation data from The Cancer Genome Atlas database (TCGA). MS-HRM results (**a**, **c**, **e**) of recent- and archived FFPE samples, together with beta values of the corresponding genomic regions estimated by 450 BeadChip array (**b**, **d** and **f**) were visualized on box plots. Probe IDs of HumanMethylation450k bead array presented: cg22403344 (*MAL*), cg04255616 (*SFRP1*) and cg25185173 (*SFRP2*). N: normal sample; T: tumor sample; Qiagen: samples isolated with QIAamp method; Roche: samples isolated with High Pure method

High Pure isolated samples were found to have higher recovery after bisulfite conversion compared to the QIAamp method. This difference could be caused by the overestimated, and in reality relatively lower DNA input to the bisulfite conversion reaction in QIAamp isolated samples due to their higher RNA content.

In general, MS-HRM results of archived FFPE samples were found to be less reliable than those of recent FFPE samples. While all reactions of recent FFPE samples were evaluable after either of the two isolation methods, in case of archived samples the ratio of successful reactions was 97.5–100% and 75–90%, with High Pure and QIAamp isolations, respectively. Moreover, the deviation of estimated DNA methylation level values of technical parallel reactions was significantly higher in the archived group. Although MS-HRM results suggest a better performance of the High Pure isolated samples, it is important to note that the relatively lower DNA input applied for the bisulfite conversion from QIAamp samples could negatively affect their MS-HRM performance.

In the recent FFPE group all normal samples were unmethylated at each gene promoter region tested, which is in harmony with literature and TCGA data. However, archived normal samples were often found to be methylated by MS-HRM. Thus, we hypothesized that the main bias of MS-HRM with archived FFPE samples is the overestimation of methylation levels, and the high deviation of parallel reactions. The ratio of samples with conflicting methylation results after QIAamp and High Pure isolation can reflect the uncertainty of MS-HRM results in archived FFPE samples. In this regard, approximately 80% of our archived MS-HRM result could be considered to be reliable. Among the analyzed tumor samples two were found to be hypermethylated at all three gene promoters tested by MS-HRM, which could be confirmed by next generation sequencing as well.

In summary, hypermethylation of *MAL*, *SFRP1,* and *SFRP2* gene promoters were reliably detected after using both FFPE DNA isolation methods, suggesting that consistent MS-HRM methylation data can be achieved from FFPE samples. However, archived (>5 years) samples showed high deviation in parallel tests, indicating the need for more parallel reactions and careful data evaluation in this sample group. The two commercially available DNA isolation kits for FFPE tissue slightly differ regarding the recovery and purity ratios, which can influence the performance of downstream DNA methylation analysis. The QIAamp kit with its higher yield can be more suitably used for recently fixed, recent FFPE tissue samples, while the High Pure kit showed more reliable results in archived FFPE tissues.

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Conflict of Interest The Authors declare no conflict of interest.

References

1. Krijgsman O, Israeli D, Haan JC et al (2012) CGH arrays compared for DNA isolated from formalin-fixed, paraffin-embedded material. Genes Chromosom Cancer 51(4):344–352. doi:10.1002/gcc.21920
2. Lyons-Weiler M, Hagenkord J, Sciulli C et al (2008) Optimization of the affymetrix GeneChip mapping 10K 2.0 assay for routine clinical use on formalin-fixed paraffin-embedded tissues. Diagn Mol Pathol Am J Sur Pathol Part B 17(1):3–13. doi:10.1097/PDM.0b013e31815aca30
3. Schweiger MR, Kerick M, Timmermann B et al (2009) Genome-wide massively parallel sequencing of formaldehyde fixed-paraffin embedded (FFPE) tumor tissues for copy-number- and mutation-analysis. PLoS One 4(5):e5548. doi:10.1371/journal.pone.0005548
4. Kalmar A, Wichmann B, Galamb O et al (2013) Gene expression analysis of normal and colorectal cancer tissue samples from fresh frozen and matched formalin-fixed, paraffin-embedded (FFPE) specimens after manual and automated RNA isolation. Methods 59(1):S16–19. doi:10.1016/j.ymeth.2012.09.011
5. von Ahlfen S, Missel A, Bendrat K et al (2007) Determinants of RNA quality from FFPE samples. PLoS One 2(12):e1261. doi:10.1371/journal.pone.0001261
6. Macabeo-Ong M, Ginzinger DG, Dekker N et al (2002) Effect of duration of fixation on quantitative reverse transcription polymerase chain reaction analyses. Modern Pathol Off J US Canadian Acad Pathol Inc 15(9):979–987. doi:10.1097/01.MP.0000026054.62220.FC
7. Solassol J, Ramos J, Crapez E et al (2011) KRAS mutation detection in paired frozen and formalin-fixed paraffin-embedded (FFPE) colorectal cancer tissues. Int J Mol Sci 12(5):3191–3204. doi:10.3390/ijms12053191
8. Jemal A, v F, Center MM et al (2011) Global cancer statistics. CA Cancer J Clin 61(2):69–90. doi:10.3322/caac.20107
9. Cancer Genome Atlas N (2012) Comprehensive molecular characterization of human colon and rectal cancer. Nature 487(7407):330–337. doi:10.1038/nature11252
10. Caldwell GM, Jones C, Gensberg K et al (2004) The Wnt antagonist sFRP1 in colorectal tumorigenesis. Cancer Res 64(3):883–888
11. Oberwalder M, Zitt M, Wontner C et al (2008) SFRP2 methylation in fecal DNA–a marker for colorectal polyps. Int J Color Dis 23(1):15–19. doi:10.1007/s00384-007-0355-2
12. Wang DR, Tang D (2008) Hypermethylated SFRP2 gene in fecal DNA is a high potential biomarker for colorectal cancer noninvasive screening. World J Gastroenterol WJG 14(4):524–531
13. Takeda M, Nagasaka T, Dong-Sheng S et al (2011) Expansion of CpG methylation in the SFRP2 promoter region during colorectal tumorigenesis. Acta Med Okayama 65(3):169–177
14. Lind GE, Ahlquist T, Kolberg M et al (2008) Hypermethylated MAL gene - a silent marker of early colon tumorigenesis. J Transl Med 6:13. doi:10.1186/1479-5876-6-13
15. Wojdacz TK, Dobrovic A (2007) Methylation-sensitive high resolution melting (MS-HRM): a new approach for sensitive and high-

throughput assessment of methylation. Nucleic Acids Res 35(6): e41. doi:10.1093/nar/gkm013

16. Kalmar A, Peterfia B, Wichmann B et al (2015) Comparison of automated and manual DNA isolation methods for DNA methylation analysis of biopsy, fresh frozen, and formalin-fixed paraffin-embedded colorectal cancer samples. Journal of laboratory automation. doi:10.1177/2211068214565903
17. Smith TF, Waterman MS (1981) Identification of common molecular subsequences. J Mol Biol 147(1):195–197
18. Gotoh O (1982) An improved algorithm for matching biological sequences. J Mol Biol 162(3):705–708
19. Luo C, Tsementzi D, Kyrpides N et al (2012) Direct comparisons of illumina vs. Roche 454 sequencing technologies on the same microbial community DNA sample. PLoS One 7(2):e30087. doi:10.1371/journal.pone.0030087
20. TCGA Research Network: http://cancergenome.nih.gov/