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Use of multilocus methylation-specific single nucleotide primer extension (MS-SNuPE) technology in diagnostic testing for human imprinted loci

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A number of diseases have been found to be linked to aberrant methylation of specific genes. However, most of the routine diagnostic techniques to detect epigenetic disturbances are restricted to single loci. Additionally, a precise quantification of the methylation status is often hampered. A considerable fraction of patients with Silver-Russell syndrome, Beckwith-Wiedemann syndrome and transient neonatal diabetes mellitus exhibit loss of methylation at further imprinted loci in addition to the disease specific ones (multilocus methylation defects, MLMD). As the currently available tests are mainly focused on single imprinted loci on different chromosomes and thereby make the detection of multilocus methylation defects time-consuming and expensive, we established methylation-specific single nucleotide primer extension (MS-SNuPE) assays for a simultaneous quantification of methylation at multiple methylated loci. We chose loci generally affected in patients with MLMD. The method was validated by screening 66 individuals with known (epi)genetic disturbances. In comparison to other methylation-specific techniques, multilocus methylation-specific single nucleotide primer extension allows the quantitative analysis of numerous CpG islands of different loci in one assay and is, therefore, suitable for the simultaneous diagnostic testing for different congenital imprinting disorders in parallel, as well as for MLMD.

Introduction

The need for reliable and simple quantitative assays to screen different imprinted loci for aberrant methylation became obvious with the growing number of patients carrying (mosaic) hypomethylation at multiple imprinted loci (multilocus methylation defects, MLMD), but specific phenotypes. Meanwhile MLMD has been reported for the transient neonatal diabetes mellitus (TNDM), the Prader-Willi (PWS), the Silver-Russell (SRS) and the Beckwith-Wiedemann syndromes (BWS).[1-9] Whereas MLMD is rare in PWS, it is a common finding in specific molecular subgroups of SRS (7%), BWS (25%) and TNDM patients. Indeed, in all MLMD cases, at least one of the two imprinting control regions (ICRs) in 11p15 shows hypomethylation; in many cases both ICRs are affected. In addition, several other imprinted loci show hypomethylation in MLMD, but a homogeneous pattern cannot be observed, not even in the same clinical cohorts. As shown by recent publications, testing for MLMD is needed for a proper diagnostic workup in patients with unusual phenotypes compatible with an imprinting disorder.[9] As a monogenic cause for MLMD has been reported in several MLMD patients,[10,11] the identification of MLMD (and the underlying genomic mutations) is important for genetic counseling, as an increased recurrence risk has to be considered in that situation. A diagnostic algorithm for imprinting disorders should therefore include tests for further imprinted loci in addition to the disease-specific ones to identify MLMD.

Several techniques for the detection of aberrant methylation at imprinted genomic loci are applied in routine diagnostics and can be performed with standard laboratory equipment (reviewed in ref. 12). In particular, methylation-specific PCR approaches (MS-PCR) and multiplex ligation-dependent probe amplification assays (MS-MLPA) are widely used in diagnostic labs because they are easy to handle and some are commercially available.[13,14] For screening approaches, MS pyrosequencing is an appropriate tool but, often, not applicable in routine diagnostics. In fact, the available assays do not cover more than 2–3 loci and are often focused on single diseases. Additionally, some of these assays are only semiquantitative and therefore do not allow a reliable detection in case of mosaic distribution of an (epi)mutation.

Recently, high throughput or ultra deep methods for the detection of methylation patterns, such as next generation bisulfite

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Table 1. MS-SNuPE results of 14 patients with different (epi)mutations at one or more loci and four control samples

	IGF2R	PLAGL1	GRB10	MEST	H19	IGF2P0	KCNQ1OT1	MEG3
Control 1	0.51	0.50	0.50	0.49	0.50	0.48	0.50	0.51
Control 2	0.50	0.50	0.50	0.49	0.49	0.54	0.50	0.50
Control 3	0.49	0.50	0.50	0.51	0.51	0.49	0.50	0.49
Control 4	0.49	0.50	0.50	0.50	0.51	0.48	0.49	0.49
MLMD-1	0.19	0.51	0.49	0.06	0.19	0.23	0.13	0.50
MLMD-2	0.23	0.50	0.50	0.50	0.33	0.32	0.50	0.41
upd(14)pat	0.52	0.51	0.52	0.49	0.51	0.52	0.50	0.82
upd(14)mat	0.49	0.50	0.52	0.52	0.50	0.51	0.51	0.09
IGF2R GOM	0.81	0.49	0.50	0.51	0.51	0.51	0.50	0.48
upd(7)mat	0.50	0.50	0.86	1,28	0.50	0.52	0.50	0.48
upd(7q)mat	0.49	0.50	0.51	1,28	0.50	0.50	0.51	0.48
SRS H19DMR LOM-1	0.51	0.52	0.52	0.53	0.42	0.41	0.51	0.49
SRS H19DMR LOM-2	0.47	0.51	0.52	0.51	0.37	0.32	0.48	0.48
SRS dup(11p15.5)mat	0.50	0.51	0.51	0.53	0.46	0.41	0.56	0.49
BWS dup(11p15.5)pat	0.51	0.51	0.52	0.51	0.55	0.60	0.42	0.47
BWS KvDMR LOM	0.50	0.51	0.51	0.51	0.53	0.54	0.15	0.50
BWS upd(11p15.5)pat	0.51	0.50	0.50	0.53	0.56	0.63	0.42	0.50
TNDM upd(6)pat	0.13	0.06	0.52	0.53	0.49	0.49	0.51	0.49
Mean	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Median	0.50	0.50	0.50	0.50	0.50	0.49	0.50	0.50
SD	0.02	0.01	0.02	0.01	0.02	0.03	0.01	0.01
Mean + 3SD	0.549	0.539	0.547	0.533	0.573	0.547	0.533	0.526
Mean - 3SD	0.453	0.460	0.451	0.469	0.429	0.431	0.472	0.473

The given values are the means of the normalized MIs obtained by two different SNuPE reactions and two primer sets (SPS-1, SPS-2). Loss of methylation is marked in green (mean - 3SD); gain of methylation is marked in red (mean + 3SD). Grey: due to dup(11p15.5) we would have expected a significant increase/decrease of MI, for further explanation see text. Mean, Median and SDs are based on three control samples independently processed in five runs (see also **Table S1**); MLMD, multi locus methylation defect; upd, uniparental disomy; GOM, gain of methylation; SRS, Silver-Russell syndrome; LOM, loss of methylation; BWS, Beckwith-Wiedemann syndrome; TNDM, transient neonatal diabetes mellitus.

sequencing or methylation specific arrays, have been developed (reviewed in ref. 15). Indeed, they provide comprehensive information about the whole epigenome but they require extensive bioinformatics workups and special laboratory equipment. The obtained information is of profound interest for scientific purposes but, for standard routine processing, they are too complex. Thus, for diagnostic purposes, tests restricted to a selection of specific loci with known imprinting status are required allowing an unambiguous interpretation.

For rapid screening of aberrant methylation of several differentially methylated regions (DMR) that have been reported to be frequently affected in MLMD patients, we established multilocus quantitative methylation-sensitive single-nucleotide primer extension (MS-SNuPE) tests.[16,17] MS-SNuPE is based on the ABI PRISM® SNaPshot® Multiplex Kit (Applied Biosystems, Darmstadt, Germany) and has previously been applied for quantitative evaluation of methylation of neighbored CpGs in the same island.[18] After bisulfite treatment of genomic DNA, locus-specific PCR is performed. For the detection of aberrant methylation patterns, primers hybridizing directly upstream to a cytosine of a differentially methylated CpG are designed ("SNuPE primer"). During a primer elongation step, uniquely labeled ddNTPs are added to the SNuPE primer corresponding to the methylation status of the analyzed CpG.

Results and Discussion

The recent identification of patients with MLMD[1-9] requires the analysis of numerous differentially methylated factors on different chromosomes in routine diagnostics. However, the currently applied methylation-specific tests are either restricted to a single locus (MS-PCR, bisulfite sequencing, MS-pyrosequencing) or do not include loci from different chromosomes but only those typically affected in specific syndromes (e.g., MLPA assays for BWS/SRS or PWS/AS).[1-9] We therefore aimed to develop a methylation-specific assay that allows a rapid, reliable and comprehensive analysis of multiple loci, preferentially those often affected in MLMD and in imprinting disorders associated with MLMD. As MS-SNuPE allows the multilocus analysis of several CpG islands in single tube reactions we chose this technique to develop a MLMD test for diagnostic purposes without requiring additional laboratory equipment.

Due to their frequent involvement in MLMD we included the DMRs of the loci *PLAGL1, IGF2R, GRB10, MEST, H19,*

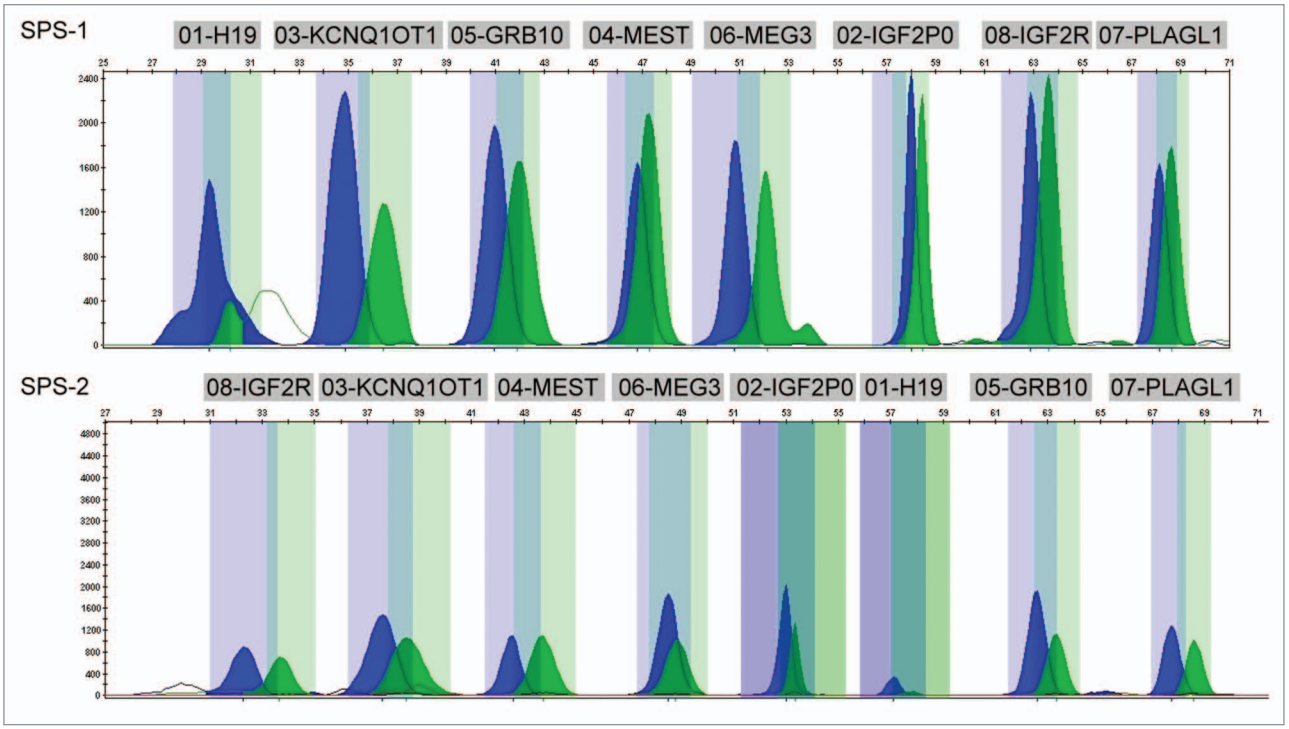

Figure 1. Raw data of the MS-SNuPE assay as displayed in the GeneMapper Software (Applied Biosystems) after the analysis with the panels for SPS-1 and SPS-2 obtained from a normal control. The methylated alleles are represented by the blue peaks, while the unmethylated alleles are displayed in green.

KCNQ1OT1, IGF2P0 and *MEG3*. The fragments were amplified by PCR in three combined PCR assays. For each locus, two CpGs were then tested for methylation by separate MS-SNuPE primers. The assays were established by analyzing 14 patients with different known epigenetic or genetic disturbances (**Table 1**), as well as four control samples (**Figs. 1 and 2**).

In all patients, the aberrant methylation patterns identified in previous tests could be confirmed. In MLMD-1 patient with SRS features, hypomethylation of the *H19* DMR was the initially detected epimutation; by MS-MLPA and MS-PCR, we furthermore identified aberrant methylation at the DMRs of *IGF2R, MEST* and *KCNQ1OT1*.[7] We now confirmed these results: with our new MS-SNuPE approach, we additionally observed that methylation was also reduced for the *IGF2P0* locus in 11p15. In a second SRS patient with MLMD (MLMD-2),[7] a slightly different methylation pattern could be identified: like in MLMD-1, the DMRs of *H19* and *IGF2P0* in 11p15 as well as of *IGF2R* revealed reduced methylation but, in MLMD-2, the *MEG3* DMR was affected. In these patients, as well as in the SRS and BWS patients with isolated loss of methylation at *H19*/*IGF2P0* and *KCNQ1OT1* (SRS *H19* LOM-1 and -2, BWS *KCNQ1OT1* LOM), respectively, methylation was not completely erased.

A methylation pattern consistent with a nearly complete hypomethylation could be demonstrated in the case of upd(14)mat for the *MEG3* DMR and for the *PLAGL1* DMR in the case of upd(6)pat. In upd(7)mat and upd(7q)mat carriers, obvious hypermethylation for the *MEST* (and *GRB10*) loci, as well as for the *MEG3* DMR in case of upd(14)pat, was detected.

As the MS-SNuPE analyses in the epimutation carriers (MLMD-1, -2; SRS *H19*-LOM-1, -2; BWS *KCNQ1OT1* LOM) showed, the range of hypomethylation is broad and, therefore, illustrates the mosaic distribution of epimutations, which is well known in SRS and BWS patients.

However, the *H19* locus did not allow an unambiguous identification of duplication in 11p15 (**Table 1**). Indeed, the MIs were at the lower [in case of dup(11p15.5)mat] or upper [in case of dup(11p15.5)pat] borders of the mean ± 3SD range, obtained from the normal controls, whereas it was clearly detectable by the other tested loci in 11p15.5. The reason for this unsatisfactory result is currently unclear.

The methylation patterns of the DMRs of *H19* and *IGF2P0* showed a direct correlation in the patients used for validation. Nevertheless, in some SRS patients, isolated hypomethylation at the *IGF2P0* locus[19,20] has been observed. These patients might be missed by the currently available MS-MLPA analysis, as the *IGF2P0* locus is not included.

We then validated the test system by analyzing 52 additional probands with known epimutations or mutations from routine diagnostics, as well as 24 healthy controls (**Table 2; Table S1**). We could confirm all previous results by the new MS-SNuPE approaches. We did not detect any additional aberrant methylation pattern in this cohort except one BWS patient with an unusual history of tumors. Initial molecular genetic testing restricted to the 11p15.5 loci indicated a paternal uniparental disomy of chromosome 11p15 [upd(11p15)pat]; with the new MS-SNuPE assay we observed a generally disturbed methylation

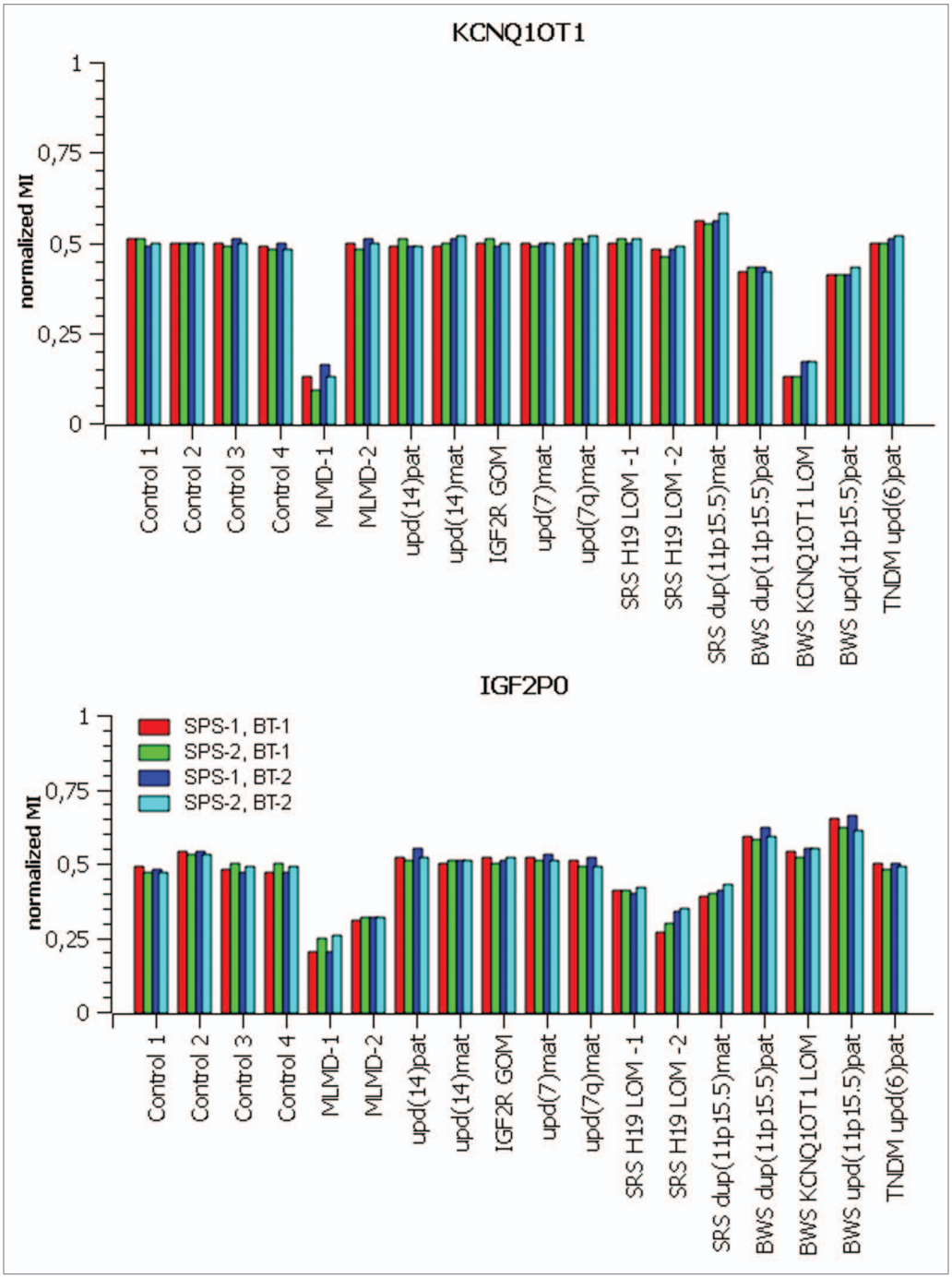

Figure 2. Normalized methylation indices (nMI) of 14 screened patients and 4 control samples for the loci *KCNQ1OT1* and *IGF2P0*. MIs are calculated as described in the material and method section. A nMI of 0.5 represents a normal methylation pattern where one allele is methylated while the other one is not. In each bar chart the two different primer mixes SPS-1 and SPS-2 for two different CpGs are compared with each other. Both primer assays were performed twice for two independent bisulfite treatments (BT-1, BT-2). (MLMD, multi locus methylation defect; upd, uniparental disomy; dup, duplication; GOM, gain of methylation; SRS, Silver-Russell syndrome, LOM, loss of methylation; BWS, Beckwith-Wiedemann syndrome; TNDM, transient neonatal diabetes mellitus).

of all tested imprinted loci. Further microsatellite-typing studies confirmed a mosaic genome wide paternal uniparental disomy, a finding that has been reported in rare cases.[21]

However, it has to be considered that the MS-SNuPE procedure, like the other techniques, does not allow distinguishing between (mosaic) uniparental disomy, deletions/duplications or isolated methylation defects. Therefore, a more detailed characterization of aberrant MS-SNuPE results using a second suitable method is implicated to ascertain the molecular diagnosis.

Reproducibility of the MS-SNuPE approaches was confirmed by (1) analyzing all samples with aberrant methylation patterns initially detected by other methods twice using DNA from independent bisulfite treatments (**Table 1; Table S1**) and (2) validating the technique in the 52 further DNA samples with known molecular alteration as aforementioned (**Table 2**) and additional 20 controls. The deviation of the MIs in different bisulfite treatments and primer elongation reactions was <10% in general. To assess the sensitivity of our MS-SNuPE assays to detect different levels of methylation, we performed mixing experiments using fully methylated and fully unmethylated EpiTect Control DNA (Qiagen) (**Fig. 3**). For all loci included in the MS-SNuPE assay, a linear correlation to the percentage of methylated DNA could be observed.

For easier comparison between different runs of the same assay, we analyzed the same three healthy controls in every run and defined the observed methylation index as normal. The use of these controls in every run is crucial as the use of a one vs. all calculation might be hampered if too many positive samples are present in one run, even if the median of all samples is used. Like in other quantitative assays like MLPA, the use of the same controls in every run allows the evaluation of the quality of single experiments. Due to the applied normalization method, a methylation index (nMI) higher than one could be observed for single loci. The non-normalized MI was always observed in the range between zero to one.

Table 2. Overview on all DNA samples used for establishment and validation of our newly developed MS-SNuPE approach

SRS		BWS		TNDM	
Disturbance	**n**	**Disturbance**	**n**	**Disturbance**	**n**
upd(7)mat	22	upd(11p15)pat	10	upd(6)pat	3
upd(7q)mat	2	dup(11p15)pat	2		
dup(11p15)mat	1	KCNQ1OT1 LOM	3		
H19 LOM	10	H19 GOM	1		
Total	35		16	Total	3
upd(14) Syndromes		**MLMD**		**Controls**	
Disturbance	**n**	**Disturbance**	**n**		**n**
upd(14)pat	1	MLMD	7	Normal Controls	24
upd(14)mat	4				
Total	5	Total	7	Total	24

In total, 66 patients with different molecular alterations were tested, these defects were initially diagnosed by MS-MLPA or single-loci MS-PCR approaches. (LOM, loss of methylation; GOM, gain of methylation)

Our data show that MS-SNuPE can be used for the rapid and reliable quantification of methylation differences in patients with congenital imprinting disorders. Several advantages of MS-SNuPE in comparison to other tests routinely used for detection of aberrant methylation are obvious:

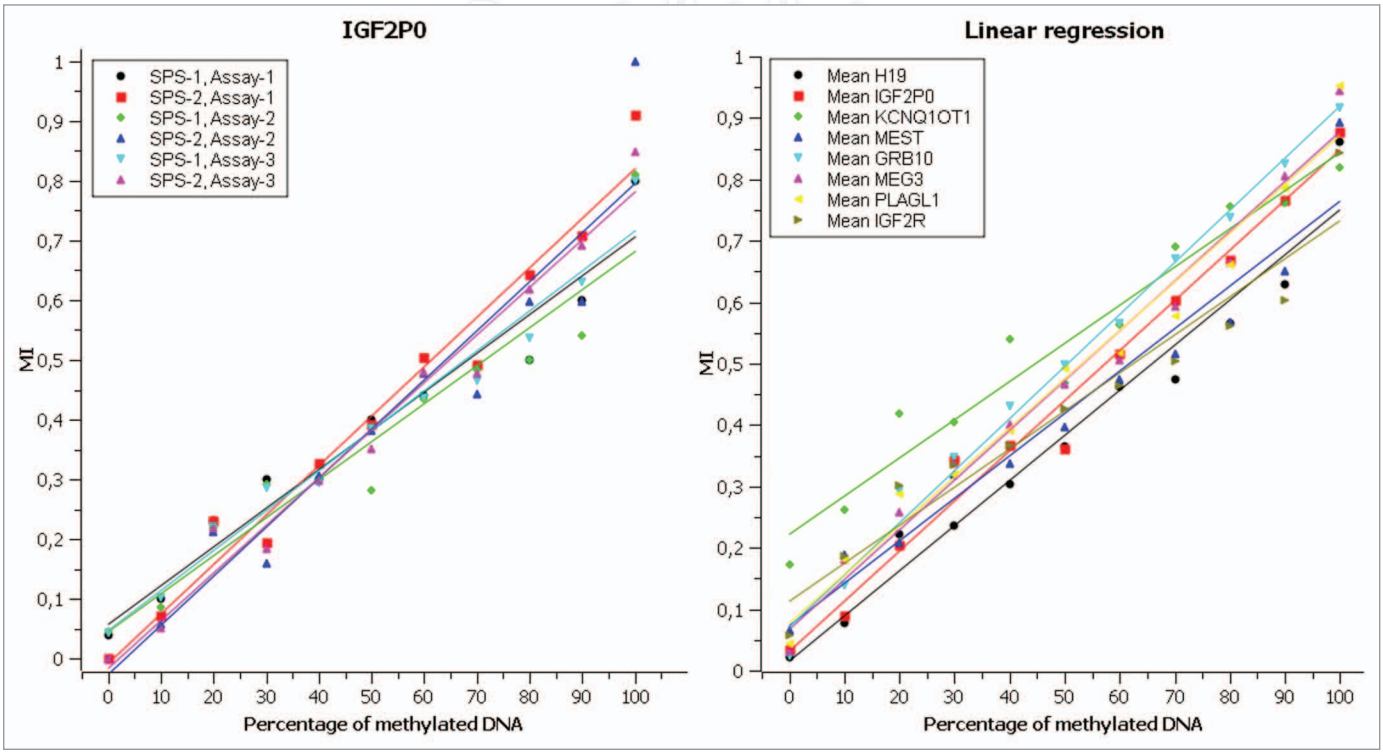

Figure 3. Non-normalized titration curves from mixing experiments with fully methylated and unmethylated DNA. Mixtures were tested ranging from 0–100% of methylated DNA in 10% steps. Both primer sets (SPS-1, SPS-2) were used for quantification in three independent experiments (Assay 1–3). The given values are the MIs calculated as the percentage of the methylated allele in relation to the total amount of DNA. (A) Titration curve for the *IGF2P0* DMR with different ratios of methylated DNA for both primer sets and three independent MS-SNuPE reactions; (B) mean values and linear regression for each locus included in the assay (*H19*, *IGF2P0*, *KCNQ1OT1*, *MEST*, *GRB10*, *MEG3*, *PLAGL1* and *IGF2R*) obtained from the two primer sets (SPS-1, SPS-2) and three independent MS-SNuPE experiments (Assay 1–3).

Table 3A. Information on primers and fragments used for MS-SNuPE assays

Locus	PCR primers	Primer sequences	nt position of PCR product#	Size of PCR product (bp)	pmol/ reaction
PCR Mix 1					
MEST	MEST_SNuPE_F*	*gat c*TY GTT GTT GGT TAG TTT TGT AYG GTT G	chr7:129,919,303–129,919,451	170	12.5
	MEST_SNuPE_R*	*cag gaa aca gct atg ac*C CAA CCA CAC CCC CTC RTT CCC ACC			
MEG3	MEG3_SNuPE_F*	*ctt gct tcc tgg cac gag* AAG AGG GAA TAG TTT TGA GAT TTT T	chr14:100,363,514–100,363,724	246	12.5
	MEG3_SNuPE_R*	*cag gaa aca gct atg ac*T AAC CCC TCA CTA ACC TTA TCA CA			
PCR Mix 2					
KCNQ1OT1	KCNQ1OT1_SNuPE_F	AAT TAG TAG GTG GGG GG	chr11:2,677,752–2,677,873	122	100
	KCNQ1OT1_SNuPE_R	CTA AAA AAC TCC CTA AAA ATC			
IGF2P0	IGF2P0_SNuPE_F	TGA GGA TGG GTT TTT GTT TGG TAT	chr11:2,125,904–2,126,158	255	2.5
	IGF2P0_SNuPE_R	TCC TCR ATC CAC CCA AAA TAA TAT			
GRB10	GRB10_SNuPE_F*	*ctt gct tcc tgg cac gag* YGY GYG TTA GGY GAA YGY GTT AGT AYG	chr7:50,817,827–50,818,094	303	5
	GRB10_SNuPE_R*	*cag gaa aca gct atg ac*T AAT CCT AAA ATT CCT ATT ATA CTC CAA AAC			
PLAGL1	PLAGL1_SNuPE_F*	*ctt gct tcc tgg cac gag* GAT AAA TGG TAG ATG TYG TGG G	chr6:144,371,150–144,371,448	334	2.5
	PLAGL1_SNuPE_R*	*cag gaa aca gct atg ac*C AAA CCR ACT CRA ATC TAC CTA			
PCR Mix 3					
IGF2R	IGF2R_SNuPE_F	ATG YGT AGT TGG AGG YGT AT	chr6:160,346,943–160,347,341	399	25
	IGF2R_SNuPE_R	TAC CTC CCC RCA CCT TTT AC			
H19	H19_SNuPE_F	AGT AGG AGY GAG GGG TTT G	chr11:1,976,432–1,977,019	588	25
	H19_SNuPE_R	ATC CRT CRA TCA CCA CCT TA			

Primers used for PCR amplification. Primers were designed to amplify both alleles, the methylated as well as the unmethylated one from bisulfite converted DNA. Some primers are 5′-tagged (marked with asterisk) for length discrimination during the electrophoresis. The tag-sequences are printed in italics. Nucleotide position refers to genome assembly hg18.

- Patients with MLMD can be identified in a single assay.
- Patients with genome wide uniparental disomy can be diagnosed.
- Patients with different imprinting disorders can be analyzed in parallel using the same assay, disease-specific tests are not necessary. Therefore, this test is more efficient than single-locus approaches, in particular in case of rare diseases where small patient numbers are relatively costly to analyze in case of single-locus analyses.
- In comparison to other routine tests, the multilocus approach requires only a small amount of patient's bisulfite treated DNA. Whereas >100 ng of DNA are necessary for conventional MS-PCR or MS-MLPA of single loci, in MS-SNuPE, the same amount allows the analysis of eight or more loci.
- With the exception of duplications in *H19*, a reliable quantification of even slight changes in methylation is possible.
- Single primers can be changed in the PCR as well as in the SNuPE reaction thereby making the technique flexible.

Furthermore, the search for MLMD is particularly helpful in patients with negative testing results for the disease-specific loci: we recently identified a SRS patient with a *H19* methylation at the lower control range but a severe *GRB10* hypomethylation. This patient might have escaped detection by routine testing restricted to the *H19* DMR, due to the low degree of aberrant methylation. The chance to detect these patients is higher with MS-SNuPE analyses for several imprinted loci than with assays focused on single loci.

In summary, MS-SNuPE represents an adequate and economic method for the detection of aberrant imprinting and can replace the currently used routine tests for specific imprinting disorders. Indeed, in the future, high throughput techniques will allow a more comprehensive overview on the whole epigenome but, as long as the interpretation of data remains difficult to handle and the assays require additional laboratory equipment, tests restricted to specific loci with known imprinting status should be applied in routine diagnostics.

Table 3B. Information on primers and fragments used for MS-SnuPE assays

Locus	SNuPE Primers	Size of SNuPE product (bp)	nt position of the primer#	pmol/ reaction
SNuPE Primer Set 1 (SPS-1)				
H19_SPS1_R	ATA TAA ATC ACC ACT ACC RCC TCT C	26	chr11:1,976,838–1,976,862	6
KCNQ1OT1_SPS1_R*	*ttt* RAC RAC CRT TCT ACC TAA AAA CTA CRA CAA C	35	chr11:2,677,789–2,677,819	0.5
GRB10_SPS1_R*	*ttttttttttttt* AAC RAT AAC RCR ACA TCC CAC CC TC	40	chr7:50,817,894–50,817,918	0.5
MEST_SPS1_R*	*ttttttttttttttttt* CAC CAT AAC CRC RTT ATC CCA TAC C	45	chr7:129,919,372–129,919,396	0.5
MEG3-DMR_SPS1_R*	*ttttttttttttttttttttttt* AAA CCA CRA CRC AAA AAC CRA CCC C	51	chr14:100,363,569–100,363,593	0.35
IGF2P0_SPS1_R*	*ttttttttttttttttttttttttttt* AAC CAC RCC RTC CCT CAC TAA CCT C	55	chr11:2,126,076–2,126,100	0.5
IGF2R_SPS1_R*	*ttttttttttttttttttttttttttttttt* TAC CCR CCR CCT CRC CRC RCC CCT C	61	chr6:160,346,988–160,347,012	6
PLAGL1_SPS1_R*	*ttttttttttttttttttttttttttttttttttt* CRA CRC AAC CAT CCT CTT AAC TAC C	66	chr6:144,371,185–144,371,209	1
SNuPE Primer Set 2 (SPS-2)				
IGF2R_SPS2_R	CRC CTC CCT ATA CCC TAC ATA CCC C	26	chr6:160,347,253–160,347,277	0.25
KCNQ1OT1_SPS2_R*	*tt* CTC CRA CTA CCC CCR CCR CTA CCR ACR TAA C	34	chr11:2,677,821–2,677,851	0.5
MEST_SPS2_R*	*ttttttttttttt* RCC ACA ACR TTA CAA AAC ACC RAA C	40	chr7:129,919,343–129,919,367	0.5
MEG3_SPS2_R*	*ttttttttttttttttt* TCT ACR ACC ACT CCR CAA TAA ATC C	45	chr14:100,363,540–100,363,564	0.35
IGF2P0_SPS2_R*	*ttttttttttttttttttttttt* TTT CCC CAA AAA ACA CAA CCA CRC C	51	chr11:2,126,092–2,126,116	0.35
H19_SPS2_R*	*ttttttttttttttttttttttttttt* CAA CCA CAA CCR ATT CTA TAC CAT C	55	chr11:1,976,880–1,976,904	2
GRB10_SPS2_R*	*ttttttttttttttttttttttttttttttttt* RCT CCT CAA AAA CRC CCA ATC CCT C	61	chr7:50,818,015–50,818,039	1
PLAGL1_SPS2_R*	*ttttttttttttttttttttttttttttttttttttt* ACR AAA CCT CCT CCT ACC ACR TAA C	66	chr6:144,371,288–144,371,312	1

Primers for the primer elongation reactions combined in two mixes (SPS-1, SPS-2). Each SNuPE primer set contains one primer for each locus. Some primers are 5'-tagged (marked with asterisk) for length discrimination during the electrophoresis. The tag-sequences are printed in italics. Nucleotide position refers to genome assembly hg18.

Materials and Methods

Using our newly developed MS-SNuPE approach we screened a cohort of 66 patients that exhibit aberrant methylation at one or more of the selected loci, (segmental) uniparental disomy (UPD), deletions or duplications involving regions with DMRs, some of them have been reported previously in reference 7 (**Table 2**). All (epi)mutations, UPDs and imbalances have been initially identified by other methods, i.e., MS-PCR, MS-MLPA, microsatellite typing and/or microarray analysis. Furthermore, DNA samples of 24 healthy German controls were analyzed. Ethical approval was granted by the Ethics Committee of the RWTH University Hospital.

Genomic DNA was isolated from peripheral blood leukocytes using a simple salting out procedure or with the QIAamp DNA Blood Mini Kit (Qiagen). Genomic DNA was bisulfite converted using the EZ DNA Methylation Gold Kit from Zymo Research. Up to 500 ng DNA were used for bisulfite treatment and eluted in 15 μl. Each sample was bisulfite treated twice (BT-1 and BT-2). For validation mixing experiments with EpiTect Control DNA,

methylated and EpiTect Control DNA, unmethylated (Qiagen) were performed.

We established MS-SNuPE assays for analysis of the DMRs of the following loci: *PLAGL1* (6q24), *IGF2R* (6q25), *GRB10* (7p13), *MEST* (7q32), *H19* (11p15), *KCNQ1OT1* (11p15), *IGF2P0* (11p15) and *MEG3* (14q32). As one MS-SNuPE primer allows the characterization of only one single CpG we decided to design two different MS-SNuPE primers for each locus. These primers were used in two independent primer elongation assays to verify the obtained results. All primers were chosen to be antisense.

Multiplexed PCR. For amplification of the DMRs of interest, three (multiplex) PCRs with primers for up to four loci each were performed. The PCR primers were designed to amplify the methylated as well as the unmethylated alleles. PCR products were analyzed by gel electrophoresis to assess the formation of primer dimers and the amplification of all desired products. Several modifications of primer sequences were tested; the optimal primer sequences and mixes for PCR finally used for validation are listed in **Table 3A**.

Approximately 100 ng of bisulfite-treated DNA were amplified in a volume of 25 μl using the QIAGEN Multiplex PCR Kit (QIAGEN) according to manufacturer's instructions. The PCR was performed at an annealing temperature of 56.5°C for 35 cycles.

From each multiplex PCR product 1–2 μl were pooled and purified using 2 μl ExoSap-IT (usb/Affymetrix) according to the manufacturer's instructions.

Primer elongation/SnuPE. After PCR amplification of the regions of interest, two different primers for each locus were established for quantification of methylation at two distinct CpGs in each DMR. The SNuPE primers were combined to SNuPE primer set 1 (SPS-1) or set 2 (SPS-2), the optimal primer sequences and mixes for primer elongation finally used for validation are listed in **Table 3B**.

For the MS-SNuPE reaction, the ABI PRISM® SNaPshot® Multiplex Kit (Applied Biosystems) was used. A volume of 3 μl of the pooled and purified PCR products was processed in a final volume of 12 μl according to the manufacturer's instructions with an annealing temperature of 50°C for the elongation primers.

The extended primers were dephosphorylated to avoid an unspecific incorporation of additional bases by adding 1 Unit of shrimp alkaline phosphatase (usb/Affymetrix) to the mix and incubation for one hour at 37°C.

Quantification. For fragment analysis 0.5 μl of a 1:5 dilution of the dephosphorylated products and 0.1 μl GeneScan LIZ120 standard (Applied Biosystems) were dissolved in a total volume of 10 μl with formamide (Applied Biosystems). The samples were denatured for 3 min at 95°C and put on ice immediately.

The products were analyzed with LIZ120 standard on an AB3130 sequencer using a modified fragment analysis protocol. AB3130 Genetic Analyzer was set up for MS-SNuPE using the SNaPshot® Matrix Standard Set DS-02 (Applied Biosystems).

For quantification we calculated the methylation index (MI) for every analyzed CpG using the peak areas of the methylated and unmethylated allele as determined by the GeneMapper software (Applied Biosystems) [MI = methylated allele/(methylated allele + unmethylated allele)]. The mean value of the MIs of three simultaneously processed control samples was set to 0.5. The MIs of the patients were normalized against these mean values (nMI).

False-positive results were excluded by Sanger sequencing of the PCR and SNuPE primer binding sites.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/19719

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