European Journal of Human Genetics (2008), 1 – 7

www.nature.com/ejhg

ARTICLE

Determination of *KCNQ1OT1* and *H19* methylation levels in BWS and SRS patients using methylation-sensitive high-resolution melting analysis

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Beckwith–Wiedemann syndrome (BWS) and Silver–Russell syndrome (SRS) are caused by imprinting defects on chromosome 11p15.5. Standard diagnostic tests for these syndromes include methylation analysis of the differential methylated regions of the *H19* and *KCNQ1OT1* genes. Traditionally this has been conducted by Southern blot analysis. PCR-based methods greatly improve the turn around time of the test and require less DNA. One of the newly emerging techniques for SNP genotyping and mutation scanning, high-resolution melting (HRM) analysis, has been shown to be also applicable for methylation analysis. We tested methylation-sensitive HRM analysis as a method for the detection of methylation defects in a group of 16 BWS and SRS patients with known methylation status (determined previously by Southern blotting), as well as 45 normal controls. HRM analysis was able to detect all methylation aberrations in the patients and appeared to be more sensitive than Southern blotting. Variation in normal controls is minimal and the presence of SNPs in the amplified fragment does not influence the outcome of the test. We conclude that methylation-sensitive HRM analysis is a robust, fast, sensitive and cost effective method for methylation analysis in BWS and SRS.
European Journal of Human Genetics advance online publication, 15 October 2008; doi:10.1038/ejhg.2008.197

Keywords: Beckwith–Wiedemann syndrome; Silver–Russell syndrome; methylation; imprinting; methylation-sensitive high-resolution melting analysis

Introduction

The Beckwith–Wiedemann syndrome (BWS, MIM130650) and Silver–Russell syndrome (SRS, MIM180680) are caused by disturbed imprinting at 11p15. This region harbours two independently regulated clusters of imprinted genes. The first cluster contains the *IGF2* and *H19* genes and is under the control of the imprinting centre 1 (IC1) upstream of the *H19* promoter. This IC is differentially methylated and methylation is only present at the paternal allele. The second cluster contains, among others, the *CDKN1C* and *KCNQ1OT1* genes and is controlled by imprinting centre 2 (IC2) overlapping the *KCNQ1OT1* promoter. This region is methylated on the maternal allele only.

The Beckwith–Wiedemann syndrome is an overgrowth syndrome. A small percentage of the patients have a mutation in the *CDKN1C* gene, whereas the majority of the patients display a methylation defect, namely, hypermethylation of IC1, hypomethylation of IC2, or both. Aberrant methylation of both ICs is usually caused by a (mosaic) paternal UPD or, in a few cases, by a paternal duplication of 11p15.[1]

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Received 18 June 2008; revised 10 September 2008; accepted 17 September 2008

Silver–Russell syndrome is a growth retardation syndrome and is genetically heterogeneous. In a subset of patients (30%) hypomethylation of IC1 is found,[2–4] which is opposite to the aberrations found in BWS patients.

Molecular genetic analysis for confirmation of a clinical diagnosis of BWS and SRS is done by methylation analysis of both IC1 and IC2. The methylation defects in BWS and SRS are mosaic and therefore the test for methylation must be quantitative. In the past, this was done by Southern blotting using methylation-sensitive enzymes and measurement of the intensity of the bands representing the methylated and unmethylated allele. Methylation indices were scored aberrant when methylation indices are <0.4 or >0.6.[5] This method requires a large amount of DNA and is very time consuming. Therefore, the use of PCR-based methods is preferred. These methods include methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA), methylation-specific PCR (MS-PCR) and pyrosequencing.[6–12] For most PCR-based methods (except MLPA) the DNA is modified with bisulphite, which converts unmethylated cytosines to uracil, inducing sequence differences between methylated and unmethylated DNA.

High-resolution melting (HRM) analysis is based on differences in melting profiles of fragments differing up to only 1 bp and is therefore able to detect methylation differences in amplicons derived from bisulphite-modified DNA.[13–16] The advantages of this method are that it is fast, cost effective and requires no post-PCR handling. Wodjacz *et al*[16] described an HRM-based test to determine the methylation status of the *H19* gene (IC1). However, aberrant methylation of IC1 is only present in approximately 25% of the BWS patients and the majority of BWS cases are caused by hypomethylation of IC2. We developed an HRM assay to assess the methylation status of both IC1 and IC2, which makes this assay suitable for the detection of all methylation defects found in BWS and SRS patients.

Methods

HRMA

To validate the method a group of 16 patients with different levels of IC1 hyper- or hypomethylation and IC2 hypomethylation were analysed by HRM. The methylation indices ranged from 0.38-0 and 0.63–0.98 as determined earlier by Southern blotting.[5] This group consisted of eight patients with a UPD (aberrant methylation of both IC1 and IC2), five patients with hypermethylation of IC2, and three patients with hypermethylation of IC1 (SRS). In addition, a set of 45 normal controls was analysed to determine the normal methylation variation in this test.

Primers were developed that amplify both methylated and unmethylated DNA in the DMRs of the *KCNQ1OT1* (IC2) and *H19* (IC1) genes. *KCNQ1OT1* (IC2) primers were LIT1_1F TTGGTAGGATTTTGTTGAGG and LIT1_1R CAA CCTTCCCCTACTACC corresponding to bases 254 969–254 988 and 255 127–255 109 of sequence AJ006345.1 (Genbank) respectively. *H19* (IC1) primers were H19_5F ATGTAAGATTTTGGTGGAATAT, H19_5R ACAAACTCACA-CATCACAACC, corresponding to bases 8242–8222 and 7971–7996 of sequence AF125183.1 (genbank) respectively. These primers amplify specifically the 6th CTCF-binding site. Specificity of the primers was confirmed by sequencing of the PCR products.

DNA was treated with bisulphite using the EZ DNA methylation Kit (Zymo research, Orange, CA, USA) according to the manufacturer's instructions. This was followed by PCR amplification, in duplicate, of 1 μl bisulphite treated-DNA in the presence of 50 mM Tris pH 9, 56 mM $(NH_4)2SO_4$, 2% DMSO, 1.5% Tween 20, 1 M Betaine, 2.5 mM $MgCl_2$, 0.125 mM dNTPs, 0.2 pmol (IC1) or 0.4 pmol (IC2) forward and reverse primer, 0.5 U *Taq* DNA polymerase (Supertaq, SphaeroQ, Leiden, The Netherlands) and 1 × LCGreen^{plus+} (Idaho Technologies, Salt Lake City, UT, USA). The PCR conditions were 95°C for 3 min, followed by 35 cycles of 95°C 20″, 55°C (IC1) or 58°C (IC2) 20″, 72°C 30″, and a final elongation step of 5 min at 72°C.

Products were melted from 65 to 98°C using the Light-Scanner System (Idaho Technologies, Salt Lake City, UT, USA). Data were analysed using the dedicated LightScanner software. Normalization regions, to correct for differences in the amount of PCR product per sample, were set before and after the major fluorescent decrease representing the melting of the PCR product. They were in the range of 78–80.5°C and 88–90°C for IC1, and 79–80°C and 87–89°C for IC2. The curve shift function brings together all the curves at a single point to compensate for small temperature variations across the plate. Shift levels were set at 0.95 for IC2 and at 0–0.05 for IC1, depending on the methylation level of the sample with the lowest methylation level in the test. The curve shift level of IC1 must be below the level of the plateau phase of the sample with the lowest methylation level to prevent erroneous alignments. The sensitivity level used for grouping of the samples was set at a level that results in all the normal control samples being included in a single group (grey colour).

Sequencing

Sequencing of the regions analysed by HRM was performed by amplification of the genomic untreated DNA with primers LIT1gF CACCGGGAGAATCGTGCT and LIT1gR AGGACACGGCACATCACTTT for IC2 and H19gF AGT TGTGGAATCGGAAGTGG and H19gR GAGCTGTGCTC TGGGATAGA for IC1 comprising the binding sites of the primers used for HRMA. Subsequent sequencing of the fragments was done using the Bigdye kit v1.1 (Applied Biosystems, Foster City, CA, USA) run on an ABI3700

sequencer (Applied Biosystems, Foster City, CA, USA) and analysed using codoncode aligner (Codoncode Corporation, Dedham, MA, USA).

Confirmation of UPD

Presence of UPD was analysed by PCR amplification of microsatellite markers or analysis of SNPs in the 11p15 region on DNA from the patients and their parents. PCR amplification of microsatellite markers D11S1288, TH and D11S988 was done using either FAM6 or HEX-labelled primers. Products were run on an ABIPrism 310 sequencer (Applied Biosystems, Foster City, CA, USA) and analysed using genemapper software (Applied Biosystems, Foster City, CA, USA).

Twelve SNPs, rs16928285, rs11022922, rs3864884, rs2023818, rs1811815, rs800338, rs800337, rs800336, rs739677, rs7951832, rs739502 and rs163150 were analysed by direct sequencing (primers available on request).

Results

In normal individuals the methylation ratio of the differentially methylated regions in imprinted genes is 0.5. The contribution of the methylated allele derived from one parent is equal to the contribution of the unmethylated allele derived from the other parent. We analysed the methylation status of the 6th CTCF-binding site of the *H19* DMR (IC1) and the DMR of *KCNQ1OT1* (IC2) in 16 BWS or SRS patients with known methylation defects using HRM analysis. HRM measures the fluorescence signal of an intercalating dye in double-stranded DNA. When the DNA melts, the dye is released and the intensity of the signal drops. Melting profiles of both IC1 and IC2 amplicons derived from bisulphite-modified DNA of normal controls showed two melting domains, caused by the relatively early melting of the unmethylated allele (containing more AT bonds) and the later melting of the methylated allele. The two domains are separated by a plateau phase (Figure 1a and b). The position of the plateau at the vertical scale represents the ratio between the unmethylated and the methylated allele. In case of hypomethylation, there is an increased contribution of the unmethylated allele resulting in a relatively longer melting phase of the unmethylated allele, which results in a plateau phase at a lower fluorescence level. Similarly, hypermethylation results in a relatively small contribution of the unmethylated allele, leaving the plateau phase at higher fluorescence. All patients with aberrant methylation, as determined by Southern blotting, showed melting profiles different from the normal controls and the degree of deviation was consistent with the degree of hypo- or hypermethylation as determined by Southern blotting (Table 1, Figure 1). Duplicate curves overlapped. One patient (B13) was thought to have hypomethylation of IC2 only, as determined by Southern blotting. However, HRM analysis also showed hypermethylation of IC1, suggesting the presence of a UPD. This was confirmed by analysis of microsatellite markers in the 11p15 region (Figure 2a).

The group of 45 normal controls showed little variation. The patient with the lowest level of hyper- and hypomethylation (B13) was clearly different from the control population (Figure 3a and b).

After implementation of this technique in routine diagnostics, a patient B702 was identified with a slight but consistent hypomethylation of IC2 and hypermethylation of IC1, indicative of a very low percentage UPD cells (Figure 3c and d). The patient with the lowest level of UPD in the positive control population, B8, has methylation indices of 0.38 (IC2) and 0.64 (IC1), which corresponds to a UPD percentage of $\sim$25%. B702 thus must have a UPD percentage considerably lower than 25%. UPD analysis using microsatellite markers was inconclusive and is probably not sensitive enough to detect such small aberrations. Therefore, we analysed 12 SNPs in the 11p15 region by direct sequencing. Patient B702 was heterozygous for seven SNPs. The ratio of the two alleles in this patient was slightly but consistently different from the ratio seen in normal controls. In all informative cases (6) the over-represented allele was derived from the father (Figure 2b), which makes the presence of a low percentage UPD plausible. This finding demonstrates the sensitivity of the HRM method.

Although the plateau phase in the melting curves of all the normal control samples is at the same fluorescence level, the melting curves do sometimes show differences. For IC1 the normal controls can be divided in two different groups and for IC2 one sample had a differently shaped curve (Figure 3e and f). This might be caused by the presence of SNPs in the analysed fragments. No common SNPs are present in the fragment amplified from IC2, but a previously unknown variant g.255083G>A (genbank accession no. AJ006345.1) was identified in the sample showing a different melting pattern (Figure 3e). In the IC1 fragment, three SNPs are present, rs4930098 (G/C), rs2107425(C/T) and rs2071094 (G/T). Rs2107425 is a C/T polymorphism and does not change the DNA after bisulphite conversion.

The remaining two SNPs are in complete LD in all individuals tested and the normal control samples are either homozygous for the rs4930098 C allele in combination with the rs2071094 T allele, homozygous for rs4930098 G allele in combination with the rs2071094 G allele, or heterozygous for both SNPs. Homozygous rs2071094C/rs4930098T (TT after bisulphite conversion) individuals all show melting pattern 1, all rs2071094G/rs4930098G homozygous individuals show melting pattern 2 and the heterozygous individuals show either pattern 1 or pattern 2, probably depending on the parental origin of the genotype (Figure 3f). Although the SNPs do alter the

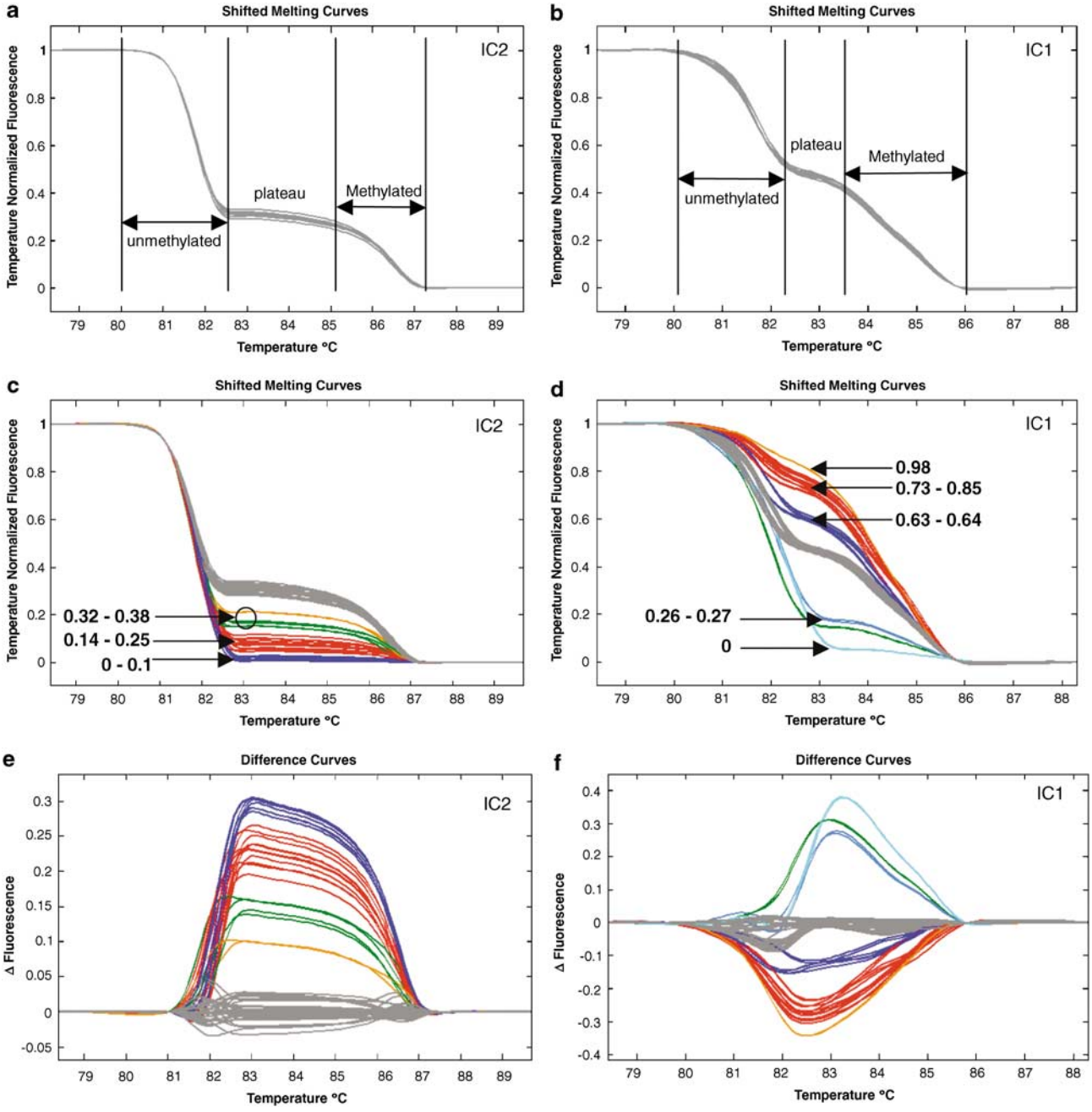

Figure 1 (**a** and **b**) Melting curves of IC2 (**a**) and IC1 (**b**) amplicons show a relative early melting of the unmethylated allele, followed by a plateau phase, and a later melting of the methylated allele. (**c**–**f**) HRMA results of the BWS/SRS patient group. Melting curves of the BWS/SRS patient group and eight control samples (in duplicate) for IC2 (**c**) and IC1 (**d**) as well as the corresponding difference curves (**e** and **f**) are shown. The grouping is as calculated by the LightScanner software and the sensitivity is set such that all normal controls are coloured alike (grey). The corresponding methylation indices of the patients as determined by Southern blotting are indicated.

shape of the melting curves, they do not change the position of the plateau that represents the ratio of methylated and unmethylated DNA. As methylation testing is based on changes in the position of the plateau phase, these SNPs do not interfere with the assessment of the methylation state.

Discussion

In the BSW/SRS patient group included in this study, all aberrant methylation patterns previously identified by Southern blotting were confirmed by HRM analysis and the degree of hypo- or hypermethylation found was comparable between the two techniques. One patient

Table 1 The results of the HRMA analysis in the group of 16 BWS/SRS patients

			IC2			*IC1*	
Patients	*Defect*	*MI*[a]	*HRMA*	*Colour*[b]	*MI*[a]	*HRMA*	*Colour*[b]
B1	UPD	0	LOM	Blue	0.98	GOM	Orange
B2	UPD	0.14	LOM	Red	0.77	GOM	Red
B3	UPD	0.17	LOM	Red	0.85	GOM	Red
B4	UPD	0.22	LOM	Red	0.82	GOM	Red
B5	UPD	0.25	LOM	Red	0.75	GOM	Red
B6	UPD	0.25	LOM	Red	0.73	GOM	Red
B7	UPD	0.32	LOM	Green	0.63	GOM	Blue
B8	UPD	0.38	LOM	Green	0.64	GOM	Blue
B9	LOM IC2	0	LOM	Blue	0.59	N	
B10	LOM IC2	0	LOM	Blue	0.59	N	
B11	LOM IC2	0.1	LOM	Blue	0.45	N	
B12	LOM IC2	0.22	LOM	Red	0.56	N	
B13	LOM IC2	0.34	LOM	Orange	**0.56**	**GOM**	**Blue**
S1	LOM IC1	0.47	N		0.26	LOM	Green
S2	LOM IC1	ND	ND		0.00	LOM	Light blue
S3	LOM IC1	ND	N		0.27	LOM	grey Blue

GOM, gain of methylation = hypermethylation; LOM, loss of methylation = hypomethylation. Discrepancies are indicated in bold.
[a]The methylation indices as determined by Southern blotting.
[b]Indicates the colour of the sample in Figure 1.

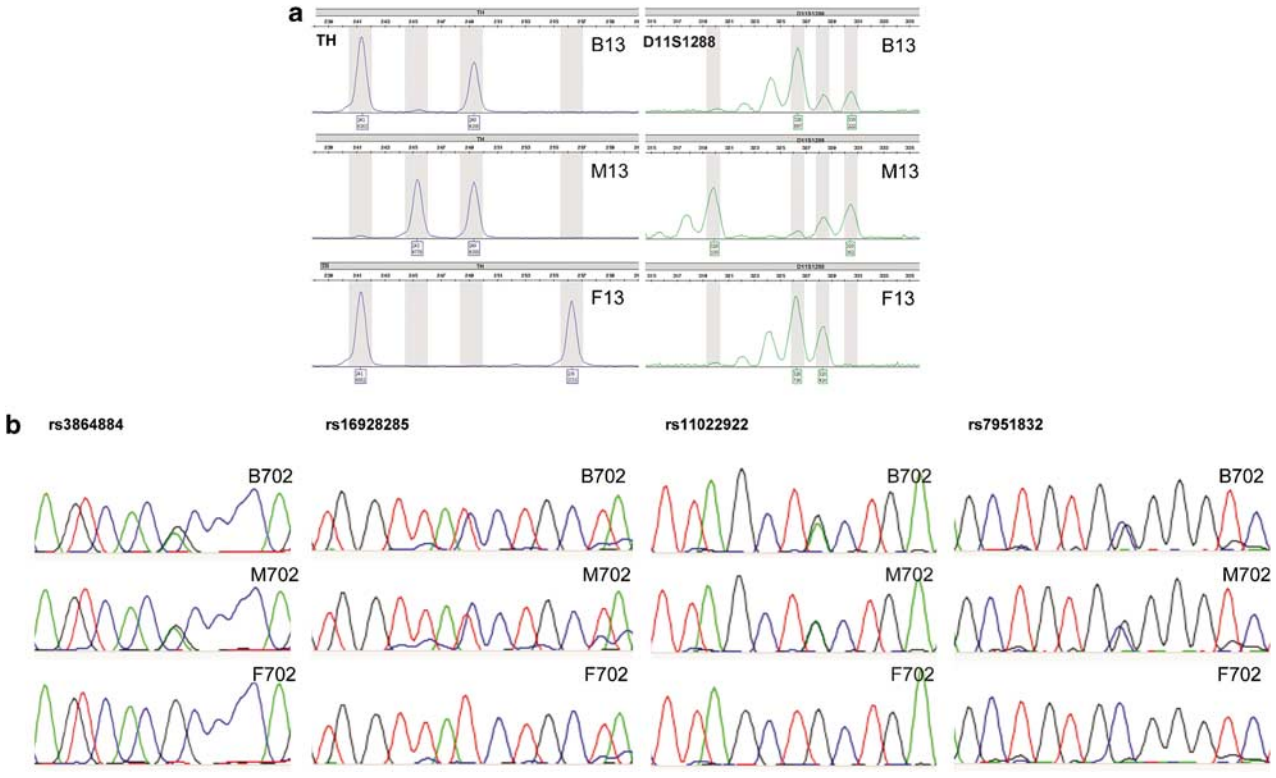

Figure 2 UPD analysis. (**a**) A mosaic paternal UPD is demonstrated with microsatellite markers TH and D11S1288 in patient B13. There is an unequal contribution of the parental allele, the paternal allele being over-represented. Electropherograms are shown for the patient (B13), his father (F13) and his mother (M13). (**b**) A low mosaic paternal UPD is demonstrated by sequence analysis of SNPs rs3864884, rs16928285, rs11022922 and rs7951832 in patient B702. The ratio between the two bases is different from normal controls and when informative, the over-represented allele is paternal.

who was found to have normal methylation of IC1 by Southern blotting showed a small degree of hypermethylation when analysed by HRMA. The hypermethylation was confirmed by the assessment of a mosaic UPD in this patient, which favors for the sensitivity of the HRMA analysis.

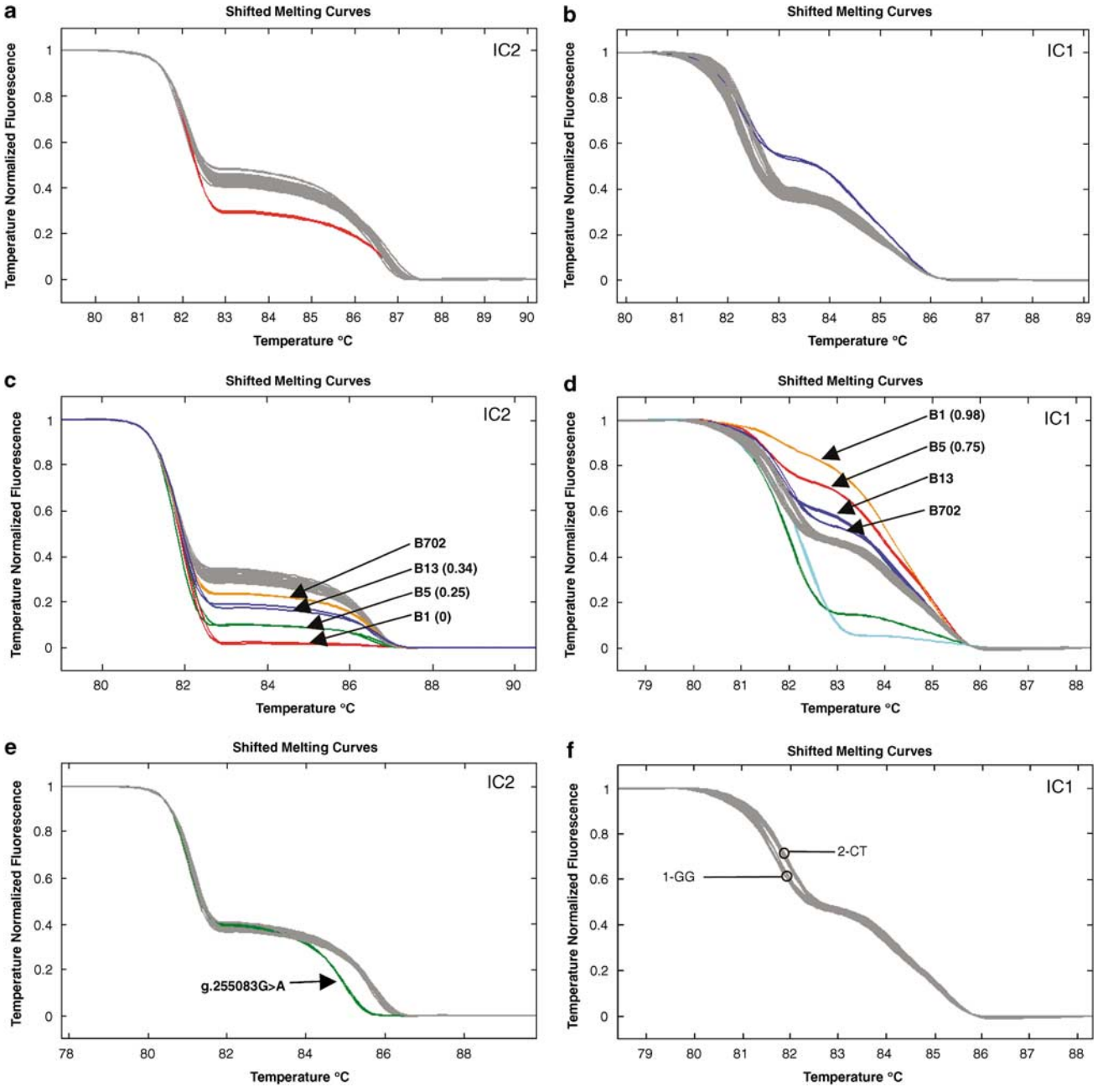

Figure 3 (**a** and **b**) Melting curves of 45 normal control individuals and patient B13 for IC2 (**a**) and IC1 (**b**). (**c** and **d**) Melting curves of patient B702 and a selection of positive controls for IC2 (**c**) and IC1 (**d**). Methylation indices of the samples are, if known, given in brackets. (**e** and **f**) Melting curves of normal control samples showing different melting patterns. The individual showing the different patterns for IC2 (**e**) carried a previously unknown heterozygous variant g.255083G > A. The differences in the curves of IC1 are caused by the common SNPs rs4930098 (G/C) and rs2071094 (G/T).

HRMA enables discriminating between fragments differing in only 1 bp.[15] The IC1 amplicon used in this study contains frequent SNPs resulting in slightly different melting patterns. Alternative amplicons, not containing known SNPs, were tested but best test results were obtained with the amplicon described here (data not shown). The position of the plateau in the melting profile, representing the ratio between the unmethylated and methylated alleles, is not altered. The presence of SNPs thus does not interfere with the accuracy of the test.

A limitation of analysis by HRM is that this system does not allow absolute quantification and calculations of

methylation indices. A range of degree of hypo- or hypermethylation is visible, but cannot be quantified. Quantification should be possible by measuring the position of the plateau phase of the patient sample relative to those of standard samples, but this will need software adaptations. Until this is available, the methylation indices of samples can be roughly estimated by comparing the profile with those of patients with known methylation status used as reference samples. In our diagnostic laboratory we implemented this technique for DNA diagnostics for BWS and SRS. To each run we add samples with known methylation indices as standards and use patient B702 as the threshold sample. Hypermethylation at IC1 or hypomethylation at IC2 more or equal to this threshold sample is considered aberrant methylation. The degree of hyper- or hypomethylation is estimated by comparing the melting profile to those of the standard samples.

In conclusion, methylation-sensitive HRM analysis proved to be a fast, reliable and sensitive method for methylation analysis as a diagnostic tool for BWS and SRS.

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