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**ALLELE-SPECIFIC METHYLATED MULTIPLEX
REAL TIME QUANTITATIVE PCR (ASMM
RTQ-PCR), A POWERFUL METHOD FOR
DIAGNOSING LOSS OF IMPRINTING OF THE
11p15 REGION IN RUSSELL SILVER AND
BECKWITH WIEDEMANN SYNDROMES**

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**ALLELE-SPECIFIC METHYLATED MULTIPLEX REAL TIME
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3 **ALLELE-SPECIFIC METHYLATED MULTIPLEX QUANTITATIVE PCR (ASMM-**
4 **QPCR), A POWERFUL METHOD FOR DIAGNOSING LOSS OF IMPRINTING OF**
5 **THE 11p15 REGION IN RUSSELL SILVER AND BECKWITH WIEDEMANN**
6 **SYNDROMES**
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Abstract

Many human syndromes involve a loss of imprinting (LOI) due to a loss (LOM) or a gain of methylation (GOM). Most LOI occur as mosaics and can therefore be difficult to detect with conventional methods. The human imprinted 11p15 region is crucial for the control of fetal growth and LOI at this locus is associated with two clinical disorders with opposite phenotypes: Beckwith-Wiedemann syndrome (BWS), characterized by fetal overgrowth and a high risk of tumors, and Russell-Silver syndrome (RSS) characterized by intrauterine and postnatal growth restriction. Until recently, we have been using Southern blotting for the diagnosis of RSS and BWS. We describe here a powerful quantitative technique, allele-specific methylated multiplex real-time quantitative PCR (ASMM RTQ-PCR), for the diagnosis of these two complex disorders. We first checked the specificity of the probes and primers used for ASMM RTQ-PCR. We then carried out statistical validation for this method, on both retrospective and prospective populations of patients. This analysis demonstrated that ASMM RTQ-PCR is more sensitive than Southern blotting for detecting low degree of LOI. Moreover, ASMM RTQ-PCR is a very rapid, reliable, simple, safe and cost effective method.

Key Words: Imprinting disorders, Beckwith-Wiedemann syndrome, Russell-Silver syndrome, 11p15 region, *H19/IGF2* ICR1 11p15 locus, *KCNQ1/CDKN1C* ICR2 11p15 locus, methylation status analysis.

Introduction

Genomic imprinting is an epigenetic mechanism by which a subset of mammalian genes are regulated. Imprinted genes are organized into clusters throughout the genome and are commonly regulated by an imprinting center region (ICR) (Edwards and Ferguson-Smith, 2007; Smith, et al., 2003). ICRs undergo parent-specific methylation on the cytosine residues of CpG dinucleotides during gametogenesis. This differential methylation regulates the allele-specific expression of several genes in the cluster (Morgan, et al., 2005; Reik, et al., 2003). A loss of imprinting (LOI) of such genes, through a gain (GOM) or a loss of DNA methylation (LOM), has been implicated in many human diseases and cancer (Robertson, 2005).

Human chromosome 11p15 encompasses two imprinted domains playing an important role in controlling fetal and postnatal growth. Each domain is differentially methylated and regulated by its own ICR [ICR1 which is paternally methylated, located in the telomeric region and regulates the *H19/IGF2* domain (OMIM: 103280/147470, respectively); ICR2 which is maternally methylated, located in the centromeric region and regulates the *KCNQ1/CDKN1C* domain (OMIM: 607542/600856, respectively)]. LOI in these two domains has been implicated in two clinical growth disorders with opposite phenotypes. LOM at ICR1 is found in more than 50% of patients with Russell-Silver Syndrome (RSS) (Gicquel, et al., 2005; Netchine, et al., 2007), whereas a GOM at ICR1 is found in 10% of patients with Beckwith Wiedemann syndrome (BWS) (Gaston, et al., 2001; Reik, et al., 1995). Abnormal DNA methylation (LOM) at ICR2 is only involved in BWS (60% of BWS cases) (Figure 1) (Gaston, et al., 2001; Reik, et al., 1995).

BWS (OMIM 130650) is characterized by pre- and postnatal overgrowth, macroglossia, abnormal wall defects, hemihyperplasia and an increased risk of childhood tumors (Cooper, et

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3 al., 2005; Gaston, et al., 2001; Schneid, et al., 1993). By contrast, RSS (OMIM 180860) is a
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5 syndrome firstly described by Silver *et al.* (Silver, et al., 1953) and Russell (Russell, 1954). It
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7 is characterized by pre- and postnatal growth retardation, a prominent forehead, relative
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9 macrocephaly, body asymmetry and feeding difficulties and/or a body mass index < -2 SDS
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11 (Netchine, et al., 2007).
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17 Our center has, for many years, been the national reference center for the molecular diagnosis
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19 and the clinical follow up of both RSS and BWS. The method we have been using for the
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21 diagnosis of RSS and BWS at our center is based on Southern blotting with methylation-
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23 sensitive enzymes, for the detection of methylation abnormalities of ICR1 and ICR2 11p15
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25 region (Gaston, et al., 2001; Netchine, et al., 2007; Schneid, et al., 1993). This technique is
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27 effective, but is time-consuming, requires large amounts of DNA and radioactivity (α ^{32}P -
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29 CTP) and involves many different steps that might introduce technical problems.
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31 Furthermore, the procedures involved take a long time and the results of the test are obtained
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33 in about 15 days.
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38 Several other methods of DNA methylation analysis have been assessed as possible
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40 replacements for Southern blotting (Fraga and Esteller, 2002). Sodium bisulfite DNA
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42 treatment followed by sequencing is one method providing detailed information about all the
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44 CpGs in a region of interest. This method generally requires cloning and transfection of
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46 bacteria that might entailing risks of contamination and false positives, and is also very time-
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48 consuming. Another alternative to this technique is pyrosequencing, which is also based on
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50 sodium bisulfite treatment of the DNA, but without the need for cloning. This technique has
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52 the advantage of being quantitative and providing information about the methylation status of
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54 each CpG in the sequence studied. Methyl-sensitive PCR has been used by several groups to
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56 study the differential methylation status of imprinted regions after sodium bisulfite DNA
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3 treatment. The fact that this technique uses one common primer and two others specific to
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5 each allele may introduce amplification bias of the methylated and unmethylated allele
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7 affecting thereby the methylation index (MI) calculation. Other quantitative techniques, based
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9 on real-time PCR, have recently been developed for detecting and quantifying changes in
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11 DNA methylation within the genome, particularly in the context of cancer (Cottrell, et al.,
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13 2004; Eads, et al., 2000; **Khulan, et al., 2006**; Thomassin, et al., 2004; Trinh, et al., 2001;
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15 Zeschnigk, et al., 2004) and for the diagnosis of **Angelman Syndrome**, RSS and BWS (Alders,
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17 et al., 2009; Bruce, et al., 2008; Coffee, et al., 2006; **Nazlican, et al., 2004**; Priolo, et al., 2008;
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19 Wojdacz, et al., 2008; Zeschnigk, et al., 2008). One of these techniques is methylation-
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21 specific multiplex ligation-dependent probe amplification (MS-MLPA) (Eggermann, et al.,
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23 2008; Priolo, et al., 2008; Scott, et al., 2008). This technique, developed for the diagnosis of
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25 RSS and BWS, can be used to analyze the methylation status of ICR1 and ICR2 on 11p15
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27 simultaneously, in the same assay. In addition, this technique offers the possibility to analyze
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29 copy number variations at the 11p15 region if the probes interrogate the abnormal region.
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39 We describe here a powerful technique — allele-specific methylated multiplex real-time
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41 quantitative PCR (ASMM RTQ-PCR) — providing results more rapidly than Southern
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43 blotting for investigating 11p15 methylation status for the diagnosis of RSS and BWS. We
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45 validated this technique by comparing the methylation indexes (MI) obtained by Southern
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47 blotting and ASMM RTQ-PCR in a control population and then carried out retrospective and
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49 prospective analyses of populations of patients. **We also compared the MI obtained with**
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51 **ASMM RTQ-PCR, Southern blotting or MS-MLPA for patients from the prospective**
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53 **population for whom we had sufficient DNA amount.**
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59 **Materials and methods**

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3 The study was conducted in accordance with national ethics regulations (authorization
4 numbers 681 and 682, *Assistance Publique-Hôpitaux de Paris*). Informed consent for genetic
5 analysis was obtained from patients or their parents and for the control **individuals**.
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8 9 10 ***Controls***

11 We used ASMM RTQ-PCR and Southern blotting to analyze the methylation status of ICR1
12 and ICR2 on 11p15 in **64 healthy individuals. Among them, 50 control subjects for whom**
13 **DNA was in sufficient quantity were also analyzed by MS-MPLA.**
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18 19 20 ***Patients***

21 We first studied two groups of patients (RSS and BWS patients) that had previously been
22 characterized clinically and molecularly. For the BWS group, all available samples (n=99)
23 collected between 2003 and 2005 from patients presenting all the clinical features of BWS
24 were eligible. For RSS, 50 samples from a previously published series of 58 RSS patients
25 were still available (Netchine, et al., 2007) and were included in this study. Methylation
26 analysis was assessed blind to the molecular diagnosis previously obtained by Southern
27 blotting.
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40 We then studied a prospective cohort (n=82) consisting of all the DNA samples from patients
41 referred to our laboratory for molecular diagnosis of RSS or BWS, over a period of nine
42 months [May 2007 - January 2008]. Methylation index (MI) was determined independently of
43 the clinical data available for each case. **Subsequently, 69 among them, for whom DNA was**
44 **still available, were analyzed also by MS-MLPA.**
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53 54 ***Southern blotting analysis***

55 We used methylation-sensitive Southern blotting to analyze the methylation status of ICR2,
56 and of ICR1 in the 11p15 region, as previously described (Gaston, et al., 2001; Gicquel, et al.,
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3 2005; Rossignol, et al., 2006). MI was calculated as follows [MI = amount of methylated
4 allele x 100/ (amount of methylated + unmethylated alleles)], with Excel software.
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10 11 12 13 ***Sodium bisulfite DNA treatment***

14 Sodium bisulfite DNA treatment converts all the unmethylated cytosine residues in CpG
15 dinucleotides to thymidine residues. The methylated cytosine residues are unaffected. This
16 process thus generates C/T polymorphisms, which can be used to distinguish between the
17 methylated and the unmethylated allele. Genomic DNA (1 µg) was treated with sodium
18 bisulfite, with the EZ DNA Methylation kit (Zymo Research, Orange, CA), according to the
19 manufacturer's instructions. It was then eluted with 50 µl RNase-free H₂O.
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31 ***TaqMan Allele-Specific Methylated Multiplex Real-Time Quantitative PCR (ASMM RTQ- 32 PCR)***

33 *Design of probes and primers*

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35 Probes and primers were designed based on sequences with the following accession numbers
36 from GenBank: NW 001838018.2 for ICR2 and AF125183 for ICR1 CTCF binding site 2
37 (CBS2) (Figure2). These two sequences encompass the *NotI* and *SmaI* sites, respectively,
38 analyzed in the Southern blotting method. Probes and primers were designed with Primer
39 Express version 3.0 (Applied Biosystems, France). We have designed two TaqMan minor
40 groove-binder (MGB) probes (TaqMan-MGB probes) specifically recognizing the methylated
41 and unmethylated alleles. The primers were designed to recognize both alleles, without
42 discrimination. We avoided the inclusion of CpG dinucleotides in the primer sequences as far
43 as possible to ensure that the methylated and unmethylated alleles were amplified equally
44 efficiently. Our TaqMan-MGB probes contained at least two C/T nucleotides, to ensure high
45 stringency, so that the hybridization of probes to each allele was very specific. The probes
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3 were stained with different fluorochromes, to make it possible to distinguish between the
4 signals of each probe in multiplex conditions: FAM for the methylated probe and VIC for the
5 unmethylated probe, referred to hereafter as “reporter dye”.
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10 11 12 *Principle underlying ASMM RTQ-PCR*

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15 The ASMM RTQ-PCR is based on the allele discrimination technology used for SNP
16 genotyping and absolute TaqMan probe quantification from standard curves, a method
17 commonly used to quantify gene expression. Following the treatment of the DNA with
18 sodium bisulfite, a C/T polymorphism is generated in the native CpG, depending on the
19 methylation status of the cytosine residue. Discrimination between the two alleles is based on
20 the use of two probes specifically recognizing the methylated allele (containing the “C”
21 residue) or the unmethylated allele (containing the “T” residue). One of the conditions for
22 TaqMan probe-based amplification is that the melting temperature (T_m) of the probe must be
23 at least 10°C higher than that of the primers, ensuring that the probes hybridize to the target
24 sequence before the primers. However, sodium bisulfite treatment decreases DNA sequence
25 stringency, limiting thereby the design of appropriate probes and primers respecting this
26 requirement. The addition of the MGB molecule at the 3' end of the probe increases the T_m of
27 the probe, permitting thereby to shorten its sequence. By designing TaqMan-MGB probes, we
28 have thus overcome the limitations imposed by sodium bisulfite DNA treatment. At steady
29 state (before the initiation of amplification), the reporter dye is close to the quencher dye,
30 resulting in the extinction of reporter dye fluorescence, principally by Förster resonance
31 energy transfer (FRET). By contrast, during primer extension (elongation step of the PCR),
32 the DNA polymerase, via its 5'-exonuclease activity, cleaves only probes hybridized to the
33 target sequence. Cleavage releases the reporter dye into the medium, where it becomes
34 fluorescent following laser excitation (Figure 3). This phenomenon occurs in each cycle of the
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3 PCR, resulting in a gradual increase in reporter dye fluorescence. Fluorescence increases only
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5 if the target sequence is complementary to the probe and is amplified during PCR. These
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7 requirements ensure that nonspecific amplification is not detected (Figure 3). The standard
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9 curves are used to determine the amount of each allele in the sample. The fluorescence
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11 intensity of each of the fluorochromes is correlated with the amount of the corresponding
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13 allele in the DNA template. Therefore, in normal conditions, we expect to have equal amounts
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15 of the two alleles (reflecting an MI of about 50%). However, in pathological conditions, in
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17 which imprinting is lost (LOI), there is an imbalance between the intensities of the two
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19 fluorochromes, reflecting LOM or GOM, depending on the imprinting abnormality
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21 concerned.
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26 *Experimental procedures*

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28 Briefly, multiplex amplification was performed with an ABI Prism 7900HT sequence detector
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30 (Applied Biosystems, France), on a 96-well plate. The reaction mixture in each well
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32 contained, in a reaction volume of 20 μ l, 10 ng of sodium bisulfite-treated genomic DNA (3
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34 μ l per well), 9 μ l TaqMan Universal Master Mix (Applied Biosystems, France), 900 nM of
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36 each primer and 200 nM of each TaqMan probe. Amplifications were carried out in triplicate.
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38 The amplification conditions, primers and probes are summarized in Table 1. The standard
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40 curve method was used to quantify each allele. A control DNA was used to generate the
41
42 standard curve, with the following dilutions: 32 ng, 10.67 ng, 3.55 ng, 1.19 ng and 0.4 ng. The
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44 results were analyzed with SDS version 2.3 software (Applied Biosystems, France) and the
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46 MI [MI= amount of methylated allele x 100/ (amount of methylated + unmethylated alleles)]
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48 was calculated with Excel software.
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55 We used EpiTect Control DNAs (QIAGEN, France) to assess the specificity and sensitivity of
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57 this technique. These DNAs have already been treated with sodium bisulfite and are totally
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59 unmethylated or methylated and ready to use. The assay was performed in triplicate, using
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3 methylated, unmethylated or a mixture of methylated and unmethylated EpiTect DNAs as the
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5 template, as described above. We mixed the two EpiTect DNAs in proportions giving a range
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7 of methylation corresponding to theoretical MIs of 100%, 75%, 60%, 50%, 40%, 25% and
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9 0%.

12 *DNA sequencing*

14 We checked primer specificity, by sequencing the two fragments corresponding to ICR1
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16 CBS2 and ICR2, using the same primers as used for ASMM RTQ-PCR, the ABI PRISM Big
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18 Dye Terminator v1.1 cycle sequencing kit and an ABI 3100 Genetic Analyzer (Applied
19
20 Biosystems). Sequences were analyzed with Sequence Navigator and Edit View software
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22 from ABI Prism.
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26 *MS-MPLA analysis method*

28 Five hundred ng of DNA from 50 control subjects and 69 patients from the prospective
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30 cohort, for whom DNA was still available, were subjected to methylation status analysis using
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32 the methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)
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34 SALSA ME030B kit (MRC Holland, Amsterdam/NL) according to the manufacturer's
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36 protocol. The MIs were calculated using the recommended Coffalyser version 8.0
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38 Directmethylation status analysis method (MRC Holland, Amsterdam/NL). The MI at both
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40 ICR1 and ICR2 were determined for each subject by the average of all MIs of target CpGs.
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48 *Statistical analysis*

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50 MIs are reported as means \pm SD. Agreement between the MI values obtained by Southern
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52 blotting and ASMMRTQ-PCR was assessed by calculating intra-class correlation coefficients
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54 (ICC) (Shrout and Fleiss, 1979) and determining the 95% confidence interval by
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56 bootstrapping. Based on the MI values obtained for ICR1 and/or ICR2, a diagnosis of RSS or
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58 BWS was established or excluded. The acceptance or exclusion of the diagnosis was treated
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60 as a qualitative value in our statistical analysis. Agreement between the two tests, in terms of

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3 this qualitative result, was assessed by calculating the kappa coefficient (Cohen, 1960). Intra-
4 class correlation coefficients and kappa coefficients indicate the degree of similarity between
5 the results obtained with the two methods. An ICC or kappa coefficient greater than or equal
6 to 0.9 was considered excellent.
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12 Bland and Altman plots were generated to check that within-subject repeatability was not
13 related to the size of the MI (Bland and Altman, 1986). Difference ($MI_{ASMMRTQ-PCR} - MI_{Southern}$
14 blot) against the average of MI ($(MI_{ASMMRTQ-PCR} + MI_{Southern\ blot})/2$). “Limits of agreement” is
15 defined by the mean difference (Cohen, 1960) and the standard deviation of the difference as
16 $dm \pm SD$. Descriptive analysis was carried out and a weighted kappa coefficient was
17 calculated with the SAS V9 System (SAS Institute, Cary, NC, USA). The ICC and its 95% CI
18 were calculated and Bland and Altman plots were generated with R software version 2.0 (R
19 Foundation for Statistical Computing, Vienna, Austria; www.R-project.org).
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33 34 **Results**

35 36 *Development of the ASMM RTQ-PCR technique*

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38 Before analyzing the methylation status of DNA from patients and controls, we checked the
39 specificity of primers and probes and evaluated the sensitivity of this technique.
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43 44 *Primer specificity*

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46 The specificity of the primers was evaluated by direct sequencing of the amplification product
47 of CBS2 ICR1 and ICR2 of a control DNA sample with the same primers used in the ASMM
48 RTQ-PCR experiment. The expected C/T dinucleotide polymorphisms were identified at the
49 CpG dinucleotides for both CBS2 ICR1 and ICR2 sequences (Supp. Figure S1). These results
50 demonstrate the high specificity of the primers and the efficacy of sodium bisulfite DNA
51 treatment.
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59 60 *Efficiency, specificity and sensitivity of the ASMM RTQ-PCR*

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3 The standard curves for the methylated and unmethylated probes were used to calculate the
4 efficiency of the assay. The amplification efficiencies of CBS2 ICR1 and ICR2 were 98% and
5 90%, respectively, and the correlation coefficients (R^2) exceeded 99% for both regions. These
6 results demonstrate the high amplification yield of our assay (Supp. Figure S2).
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12 We checked the specificity of probe hybridization to each allele and investigated whether our
13 technique could detect subtle differences in methylation, using EpiTech control DNAs. These
14 control DNAs are of two types: totally unmethylated or totally methylated. The probes for
15 quantifying the CBS2 ICR1 and ICR2 regions were highly specific, with no cross
16 hybridization between the two alleles. The methylated EpiTect DNA is obtained
17 enzymatically, and displays about 95% methylation, according to the manufacturer. Our
18 results for the methylated probes for ICR2 were consistent with this value (94%). The MIs
19 calculated for the mixtures of EpiTech DNAs in various proportions were consistent with
20 theoretical expectations for the two regions (Supp. Figure S3). Thus, these results demonstrate
21 the high specificity of our probes and the high sensitivity of our assay.
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35 36 *Clinical validation*

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38 We have firstly validated ASMM RTQ-PCR by comparing it to our gold-standard method,
39 Southern blotting. Since MS-MPLA is becoming increasingly used as an analytical technique
40 for DNA methylation assessment particularly for RSS and BWS diagnosis, we secondly
41 assessed a comparison of the three methods in the prospective analysis group of patients.
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48 *Control subjects*

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50 We first analyzed the methylation of the CBS2 ICR1 and ICR2 regions of DNA from 64
51 controls, by both Southern blotting and ASMM RTQ-PCR. The MIs calculated for the control
52 subjects were normally distributed, which allowed us to calculate a mean MI and standard
53 deviation (SD). The MIs calculated for CBS2 ICR1 and ICR2 by the two methods were
54 concordant (Figure 4 A and B). The intra- and inter-assay coefficients were calculated for
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3 ASMM RTQ-PCR, and both were found to be 6% (data not shown). We considered a MI to
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5 be normal if it was within two standard deviations of the mean MI value for control subjects,
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7 and abnormal if it was outside this range (Figure 4 B).
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10 11 12 *Patients*

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15 In the retrospective study, the patients were analyzed by ASMM RTQ-PCR, blind to the
16
17 molecular diagnosis previously obtained by Southern blotting. In the prospective study, the
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19 patients were analyzed by both methods, blind to the clinical data. An example of the
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21 amplification curves of control subjects and patients with LOM at CBS2 ICR1 or ICR2 is
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23 shown in supplementary data (Supp. Figure S2).
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29 *Clinical application*

30 31 *Retrospective RSS population*

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34 In the 50 identified cases of RSS, the mean MI of CBS2 ICR1 assessed by ASMM RTQ-PCR
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36 was 28 ± 20 and that assessed by Southern blotting was 32 ± 17 . The ICC was 0.89 [0.84-0.92]
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38 (Supp. Figure S4A). For the qualitative results, 62% of samples showed LOM by both
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40 techniques, indicating maximal levels of agreement ($\kappa=1$; Supp. Table S1).
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46 *Retrospective BWS population*

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48 The mean MI of CBS2 ICR1 was 60 ± 13 by ASMMRTQ-PCR and 58 ± 12 by Southern
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50 blotting. The agreement between the two MI measurements was 0.98 [0.97 - 0.99] (Supp.
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52 Figure S4B). All normal results and all gains of DNA methylation identified by Southern
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54 blotting were also identified by ASMM RTQ-PCR, giving an agreement of 0.91 [0.82 - 1].
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56 Three cases identified as LOM by Southern blotting were identified as normal by ASMM
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58 RTQ-PCR (Supp. Table S2). These three patients all had a borderline MI (46%) on Southern
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3 blots, the lower limit being 48%. ICR1 LOM is not a molecular defect identified in BWS.
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5 Indeed, two of these patients had LOM at ICR2, while the third one had no ICR2 LOM.
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10 The mean MI for ICR2 was 22 ± 21 by ASMMRTQ-PCR and 23 ± 20 by Southern blotting.
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12 Agreement between the two MI measurements was 0.98 [0.97 - 0.98] (Supp. Figure S4C). No
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14 divergence between the two techniques was observed for qualitative results. Both identified
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16 64% of samples as LOM and 36% as displaying normal DNA methylation ($\kappa=1$; Supp.
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18 Table S3).
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22 *Prospective population*

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24 The MI values for CBS2 ICR1 and ICR2 are presented in Table 2.
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27 *CBS2 ICR1 11p15*

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29 The mean MI value for CBS2 ICR1 11p15 assessed by ASMM RTQ-PCR was 50 ± 10 , and
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31 that assessed by Southern blotting was 54 ± 8 . The quantitative agreement coefficient was 0.78
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33 [0.59 – 0.86]. A Bland and Altman plot was generated to investigate the variation between
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35 individuals (Figure 5A). We identified four extreme points resulting in a positive slope for the
36
37 overall plot, indicative of a proportional error. The MI value obtained by Southern blotting
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39 was systematically higher than the value obtained by ASMM RTQ-PCR.
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43 Three disagreements concerning the qualitative result were identified (Table 3): two GOMs
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45 and one LOM identified by Southern blotting were identified as normal by ASMM RTQ-
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47 PCR. The kappa coefficient was good, but not excellent, at 0.82 [0.63 – 1].
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51 *ICR2 11p15*

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53 The mean MI value for ICR2 11p15 was 42 ± 14 when assessed by ASMM RTQ-PCR and
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55 42 ± 13 when assessed by Southern blotting. Quantitative agreement was excellent ($\text{ICC}=0.96$
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57 [0.92 – 0.98]). Bland and Altman plots showed that most of the differences lay within the
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59 confidence interval of the difference, with the exception of three points (Figure 5B).
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3 Two disagreements in qualitative results were identified (Table 4). Two cases identified as
4 normal on Southern blotting were identified as abnormal by ASMM RTQ-PCR (both LOM).
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6 One patient was a RSS patient with multilocus LOM and the other had BWS with clinical
7 characteristics concordant with ICR2 LOM. Despite these two disagreements, the kappa
8 coefficient was good, at 0.89 [0.75 – 1].
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17 **MS-MLPA analysis results**

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19 We analyzed the methylation status of ICR1 and ICR2 11p15 region for 50 control subjects
20 and 69 patients from the prospective population for whom DNA was still available. The mean
21 and SD calculated from the control population were 55±3 % (range 49-61%) and 60±4 %
22 (range 52-68%) for ICR1 and ICR2, respectively. This range is larger than that obtained by
23 ASMM RTQ-PCR and Southern blotting (Supp. Figure S5). According to these ranges, the
24 diagnosis established with MS-MPLA is concordant in 80% of the cases (55/69) with that
25 established by ASMM RTQ-PCR and Southern blotting; whereas in 20% of the cases (14/69)
26 there is a discordance between the three methods (Supp. Table S4). However, for 71% of the
27 discordant patients (10/14), MIs obtained by MS-MLPA were discordant with those of
28 ASMM RTQ-PCR and Southern blotting. Almost all the discordant abnormal MIs obtained
29 with MS-MLPA were represented by GOM at ICR1 (n=5) or at ICR2 (n=3); only 2 slight
30 LOM were observed at both ICR1 and ICR2. Strikingly, one RSS patient and one BWS
31 patient with respectively LOM and GOM at ICR1 11p15 with ASMM RTQ-PCR and
32 Southern blotting had a normal MI with MS-MLPA (Supp. Table S4). These results
33 demonstrated that MS-MLPA results can be discordant with both ASMM RTQ-PCR and
34 Southern blotting results.
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Discussion

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3 We have developed a technique for analysis of the methylation of CBS2 ICR1 and ICR2 in
4 the 11p15 region, for the molecular diagnosis of RSS and BWS. We first validated the
5 primers and probes used, and then studied a large cohort of patients (given that both these
6 conditions are rare) and control subjects, by both Southern blotting and ASMM RTQ-PCR.
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12 Since the MS-MPLA is also used for RSS and BWS diagnosis, we secondly assessed with this
13 method a sub-group of the patients analyzed in the prospective analysis to allow a comparison
14 between the three methods.
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20 Human ICR1 in the 11p15 region contains seven CTCF binding sites (CBS) within two
21 repetitive sequences and displays a high degree of sequence identity (Frevel, et al., 1999).
22 This makes it difficult to design primers and probes specific for the CBS to be analyzed
23 (CBS2). For consistency with the analysis of 11p15 region methylation by Southern blotting,
24 we designed probes for ASMM RTQ-PCR containing the restriction sites (Netchine, et al.,
25 2007; Rossignol, et al., 2006) used over many years in the diagnosis of RSS and BWS by
26 Southern blotting (Figure 2). Direct sequencing of the amplification products and the EpiTech
27 control DNAs demonstrate that our primers and probes were highly specific to the regions of
28 interest, CBS2 ICR1 and ICR2, with no cross hybridization observed in the multiplex
29 amplification assay. Using the EpiTech control DNAs, we also demonstrated ASMM RTQ-
30 PCR to be a highly robust and sensitive assay. Methylation abnormalities generally occur as
31 mosaics (Gaston, et al., 2001; Gicquel, et al., 2005; Nazlican, et al., 2004; Netchine, et al.,
32 2007; Schneid, et al., 1992; Schneid, et al., 1993), making it very difficult to identify patients
33 with low degree of LOI. Our results indicate that the risk of false positives is lower with
34 ASMM RTQ-PCR than with Southern blotting. Moreover, the high sensitivity of this assay
35 increases the likelihood of identifying patients with low degree of LOI. We validated our
36 technique by comparing the MIs of control subjects calculated by Southern blotting (gold-
37 standard method) and by ASMM RTQ-PCR. The MI values were normally distributed, which
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3 allowed to calculate the mean and SD for normal MIs. We then carried out retrospective and
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5 prospective analyses on cohorts of patients, for further validation. We observed strong
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7 agreement between the two methods, showing that ASMM RTQ-PCR is as powerful
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9 technique as Southern blotting for methylation studies and for the diagnosis of RSS and BWS.
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11 For some patients, discordance between the MIs obtained for CBS2 ICR1 with the two
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13 methods was observed, in both retrospective and prospective populations. The MIs of these
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15 patients were borderline on Southern blots. The analysis of the methylation status at CBS3
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17 ICR1 by ASMM RTQ-PCR revealed a normal MI in agreement with those obtained at CBS2
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19 ICR1; this allowed us to make definitive diagnosis conclusion (data not shown). We also
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21 identified two patients with LOM at ICR2 by ASMM RTQ-PCR, who were considered
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23 normal on Southern blotting, in the prospective study. One of these patients was an RSS
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25 patient exhibiting multilocus imprinting defect [published elsewhere (Azzi, et al., 2009)] and
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27 the other presented clinical features of BWS consistent with ICR2 LOM. When considering
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29 the qualitative results obtained for molecular diagnosis by the two methods, the concordance
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31 was excellent in the retrospective population and good in the prospective population.
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33 However, when we consider the quantitative results obtained by calculating the MI for the two
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35 methods, the ICC obtained was smaller for CBS2 ICR1. The small discrepancy between the
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37 two methods may be accounted for by differences in the characteristics of the two methods.
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39 Southern blotting is a quantitative method requiring the hybridization of a radiolabeled probe
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41 to digested DNA transferred onto a membrane. This hybridization may generate a background
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43 signal, decreasing the accuracy of the signal intensities obtained when the bands are scanned
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45 and thus affecting MI calculation. By contrast, the ASMM RTQ-PCR is based on the
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47 quantification of fluorescence from the reporter dye after primer elongation. This fluorescence
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49 is normalized with respect to a reference dye (ROX) in the reagent mixture, the fluorescence
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3 intensity of which does not change during the PCR. The MIs calculated with the ASMM
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5 RTQ-PCR method are therefore more accurate.
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8 Several other valuable methods have been developed for methylation analysis. Some of these
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10 methods are based on sodium bisulfite DNA treatment followed by subcloning and
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12 sequencing or quantitative amplification. These methods, although allowing the analysis of all
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14 CpGs between the primers, remain laborious and are not free from contamination risks (Fraga
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16 and Esteller, 2002). Another alternative to this method is pyrosequencing, which does not
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18 require subcloning, being based instead on the direct sequencing of amplification products.
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20 This technique provides quantitative information about methylation at each CpG in the
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22 sequence of interest and is therefore potentially promising. However, it remains expensive and
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24 is not yet available for diagnostic purposes in many institutions. Other methods based on
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26 sodium bisulfite DNA treatment and quantitative amplification require efficiency optimization
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28 (Cottrell, et al., 2004; Thomassin, et al., 2004) and some are limited to analysis of the
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30 methylation status of only one allele at a time and, therefore, cannot provide accurate
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32 information (Eads, et al., 2000; Trinh, et al., 2001). Coffee *et al.* have developed a TaqMan
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34 MSP method based on quantitative PCR using Light-Cycler technology, for the molecular
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36 diagnosis of RSS and BWS. The principle underlying their method is similar to that
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38 underlying ours, but the CBS6 ICR1 probes also hybridize to sites other than CBS6 in the
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40 ICR1 11p15 region. CBS6 harbors a frequent SNP (about 50%) rs10732516
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42 (NT_009237.17:g.808447G>A) that might affect the interpretation of the results (Figure 2).
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44 Moreover, Coffee *et al.* obtained a very large normal range of MIs for both CBS6 ICR1 and
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46 ICR2 (33-65% for CBS6 ICR1 and 40-64% for ICR2) (Coffee, et al., 2006). Our ASMM
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48 RTQ-PCR method was validated on a large cohort of control subjects and the MIs calculated
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50 were more accurate, as the calculated SDs were much smaller than those obtained by Coffee
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52 *et al.* [3% in our assay rather than 6% (ICR2) and 8% (ICR1) for 1 SD in Coffee's assay].
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3 QiaGen has been developed some TaqMan assays to study the methylation status of
4 promoters of certain genes involved in cancer. The principle of their assays is substantially the
5 same as our since they use two allele-specific probes to quantify the methylated and the
6 unmethylated alleles. Our assay assesses the methylation study of imprinted loci for which the
7 methylation status is balance between the parental alleles. By contrast, QiaGen's assays are
8 developed to study the methylation changes of non imprinted promoters. The methylation of
9 such regions could be different between CpGs and could hinder the hybridization of the
10 probes which could introduce an error of MI calculation. The MI is calculated using the ratio
11 of the cycle threshold (C_T) of the methylated and unmethylated allele in the same sample. To
12 be more accurate, this calculation requires the use of a control probe in the same assay so that
13 to normalize the DNA amount. We used the standard curve to determine the MI. This method
14 of calculation (standard curve) does not need the use of a control probe for normalization,
15 since the quantity of each allele is calculated by the standard curves. For the diagnosis of RSS
16 and BWS, Wojdacz *et al* and Alders *et al* (Alders, et al., 2009; Wojdacz, et al., 2008) have
17 recently developed a methylation-sensitive high-resolution melting (MS-HRM) method for
18 the methylation analysis of the *H19* promoter. Although this method is reliable, it is however
19 difficult to identify low degree of LOI present in pUPD11, 11p duplications and 11p15
20 epimutation as these abnormalities are frequently in mosaics.

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22 Some other methods based on the use of methylation-sensitive restriction enzymes have also
23 been developed for methylation analyses. These methods focus on a single CpG, and may
24 therefore fail to detect certain individuals with low degree of LOI, necessitating additional
25 testing on proximal regions (Bruce, et al., 2008). The methylation-specific multiplex ligation-
26 dependent probe amplification assay (MS-MLPA) was also developed for analysis of
27 methylation in the 11p15 region for the diagnosis of RSS and BWS (Eggermann, et al., 2008;
28 Priolo, et al., 2008; Scott, et al., 2008). It uses a set of probes hybridizing at ICR1 and ICR2,

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3 which are hybridized to the DNA and then digested with a methylation-sensitive restriction
4 enzyme (*HhaI*) before amplification of the ligated probes. This method has been described as
5 robust, reproducible and sensitive. To test this method, we have analyzed a control population
6 and a population of patients for whom the DNA was still available and who had already been
7 analyzed with Southern blotting and ASMM RQ-PCR. Our results showed that the range of
8 normal MI in both ICR2 and *H19*DMR are larger than that obtained by Southern blotting and
9 ASMM RTQ-PCR. These large values of normal range could be explained by variation of
10 MIs at each CpG. In addition, 10 among 69 patients analyzed showed discordant MI with MS-
11 MLPA when comparing with the two other methods. Strikingly, we have failed to detect two
12 patients with 11p15 abnormalities. Some probes of the probe mix ME030B particularly those
13 hybridizing at *H19*DMR have been showed to give inappropriate MIs or copy number (Priolo,
14 et al., 2008; Scott, et al., 2008). Since these reports, the SALSA ME030B probe mix has been
15 changed. It should be noted that all the methyl-specific probes hybridizing to *H19*DMR are
16 located in the promoter region of *H19* gene except one (08745-L08765) that is located
17 between CBS6 and CBS7 (Figure 2). It is recognized that ICR1 is the imprinting centre that
18 regulates the telomeric region of the 11p15 region. Furthermore, uneven methylation has been
19 previously reported at the promoter region of *H19* (Vu, et al., 2000) and dissociation between
20 the methylation status and *H19* promoter has been reported in RSS patients (Horike, et al.,
21 2009). Improvements are therefore required in the SALSA ME030B probe set, together with a
22 careful analysis and validation of the results.
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52 ASMM RTQ-PCR is easy to perform, reproducible, robust and highly sensitive. Results can
53 be obtained in one and half days, and the analysis is very easy to monitor. In addition, the use
54 of real-time PCR reduces the handling errors associated with manual manipulation, by
55 decreasing the number of laboratory steps performed in the assay. Moreover, ASMM RTQ-
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3 PCR is highly suitable for diagnostic purposes because it is relatively cheap and requires less
4 than 10 ng of sodium bisulfate-treated DNA for triplicate assays. The use of a 96-well plate
5 makes it possible to analyze 26 patients in a single PCR. This method is the first to be
6 validated on such a large series of controls and patients with and without 11p15 LOI.
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10 Robust and reliable techniques are required for molecular diagnosis of these abnormal
11 patterns of methylation occurring as mosaics. This technique is currently transferred for the
12 diagnosis of RSS and BWS in our laboratory and substituted to Southern blotting and allows
13 accurate diagnosis with a small DNA quantity. This is the first step to allow the development
14 of an antenatal diagnosis for both RSS and BWS. In our experience, there is an increasing
15 demand for BWS and RSS antenatal diagnosis to improve the obstetrical follow-up of these
16 pregnancies and orient the medical counseling in case of severe IUGR or overgrowth
17 syndrome during the pregnancy. Because of severe intra-uterine growth retardation, RSS
18 fetuses are often delivered by the obstetricians before term. However, they then frequently
19 have neonatal complication and this could be avoided if the diagnosis was known as they
20 usually have a good tolerance to *in utero* gestation until term. Since the methylation
21 anomalies of the 11p15 region occur in a mosaic manner, this diagnosis is very delicate in
22 cases of low degree of LOI and mosaicism and requires a very reliable method that needs very
23 small amount of DNA, extracted directly from the amniocytes pellet without a step of culture.
24 ASMM RTQ-PCR technique responds to these requirements and will make possible to
25 develop such a diagnosis. Furthermore, ASMM RTQ-PCR could be adapted for studies of
26 methylation status at other imprinted loci involved in human syndromes and has been used for
27 the evaluation of seven other imprinted loci as part of a multilocus study on our cohort of RSS
28 and BWS patients with LOI at 11p15 (Azzi, et al., 2009). It could potentially be used as a
29 starting point for the design of assays for postnatal as well as antenatal diagnosis for Prader-
30 Willi syndrome, Angelmann syndrome, transient neonatal diabetes mellitus, chromosome 14

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3 related syndromes and pseudohypoparathyroidism 1b. This technique could also be adapted
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5 for methylation studies in cases of cancer and for the validation of microarray methylation
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7 studies.
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41 42 **Reference**

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Figure legends:

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Figure 1: Representation of the 11p15 region and its imprinting related to 11p15 disorders. The middle panel represents the normal situation. The 11p15 region encompasses two imprinted centers, ICR1 at the telomeric domain and ICR2 at the centromeric one. ICR1 is methylated on the paternal allele, to which CTCF does not bind, thereby allowing the shared enhancers (E) access to the *IGF2* promoters and stimulating their activation. The ICR1 on the maternal allele is not methylated; CTCF can therefore bind to this allele, acting as an insulator, preventing the activation of *IGF2* promoters by shared enhancers but permitting the

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3 activation of the *H19* promoter. ICR2 is methylated on the maternal allele where *CDKN1C*
4 and *KCNQ1* are expressed. On the paternal non-methylated allele, *KCNQ1OT1* (non coding
5 RNA) is expressed and regulates the imprinting in *cis*. Two imprinting defects can lead to
6 BWS: the first is loss of methylation (LOM) at ICR2 (upper left-hand panel) leading to the
7 biallelic expression of *KCNQ1OT1* and a loss of expression of *CDKN1C* and *KCNQ1*. This
8 abnormality accounts for 60% of BWS cases. The second is gain of methylation (GOM) at
9 ICR1 (upper right-hand panel), leading to the biallelic expression of *IGF2* (the main fetal
10 growth factor) and *H19* (non coding RNA) downregulation. This abnormality accounts for
11 10% of BWS cases. In more than 50% of RSS patients, LOM at ICR1 is involved (lower
12 middle panel). This abnormality leads to the down regulation of *IGF2* and the biallelic
13 expression of *H19*.
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32 **Figure 2: Diagram representing the 11p15 region illustrating the positions of primers**
33 **and probes used in different assays.** The open boxes on ICR1 region represent the B and A
34 repeats, the gray boxes represent the positions of the 7 CTCF binding sites (CBS). ICR2 (left
35 gray box) is located in the 10th intron of *KCNQ1* gene (hatched box). The ASMM RTQ-PCR
36 assays, designed on CBS2 ICR1 and ICR2, are illustrated by black arrows and bars; the
37 TaqMan MGB-probes encompass the methyl-sensitive sites, *Sma*I (CBS2 ICR1) and *Not*I
38 (ICR2), used in Southern blotting. Coffee's assays schematized by the gray arrows and bars
39 are designed on CBS6 ICR1 and ICR2. The positions of the restriction sites and probes used
40 in Southern blotting for both ICR1 and ICR2 are depicted. MS-MLPA probe set is shown on
41 the upper panel of the diagram for H19DMR and the bottom panel of the diagram for ICR2,
42 three of the four probes interrogating H19DMR is located within H19 promoter and only one
43 probe is located between CBS6 and CBS7. The circles indicate the CpGs sites.
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3 **Figure 3: Principle of ASMM RTQ-PCR.** The upper panel represents the sodium bisulfite DNA
4 treatment step. The methylated cytosine residues (blue stars) at the CpG dinucleotide remain
5 unchanged, whereas cytosine residues without methyl groups are converted into thymidine residues.
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7 The lower panel represents the quantitative real-time PCR step. The two probes were labeled with
8 different fluorochromes, FAM (F) and VIC (V), and specifically recognized either the methylated or
9 the unmethylated allele. After elongation, the probes were degraded and the two fluorochromes were
10 released into the medium, where they became fluorescent on laser excitation. Black circles: methylated
11 cytosines; white circles: unmethylated cytosines; NFQ: non fluorescent quencher; MGB: minor groove
12 binder.
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24 Figure 4: A) Histogram showing the mean MI of CBS2 ICR1 and ICR2 calculated by ASMM
25 RTQ-PCR and Southern blotting, for a population of control subjects (n=64). The MIs in B represent
26 the mean MI \pm SD (standard deviation). Three independent experiments were carried out. The error
27 bars in the histograms represent \pm 1 SD.
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34 Figure 5: Bland and Altman's graphical representation of the intraclass correlation coefficient
35 (ICC) and its IC95% by bootstrap. ASMM RTQ-PCR and Southern blotting agreement in the
36 prospective cohort for CBS2 ICR1 (A) and ICR2 (B) (difference of MI measurements against
37 their mean value). 'Limits of agreement' is defined by the mean difference (dm) and the
38 standard deviation of the difference as $dm \pm SD$ for CBS2 ICR1 (A) and ICR2 (B).
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Table 1. Sequences of primers and probes and amplification conditions used in ASMM RTQ-PCR

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Locus	GenBank Accession Number	Primer sequence (5'-3')	Probe sequence (5'-3')	Amplification Conditions	Fragment size (bp)
CBS2 ICR1	AF125183	CBS2 F: GTTTTGATGGTGTAGA ATTGGTTGTAG	ICR1 CBS2 M: 6FAM- TTTC <u>CGG</u> GTTACGTAAGTT-MGB	95°C x 10 min [95°C x 30 s; 55°C x 30 s; 60°C x 1 min] x 50 cycles	184
		CBS2 R: TCCCATAAATATTCTA TCCCTCACTACC	ICR1 CBS2 UM: VIC- AAATGTTTTGGGTTATGTAAGT- MGB		
ICR2	NW 001838018.2	ICR2 F: GGGTTAGTTTTTTGYG TGATGTGTTT	ICR2 M: 6FAM- ATAG <u>CGG</u> T <u>CG</u> TATTT <u>CG</u> ATAT-MGB	95°C x 10 min [95°C x 15 s; 60°C x 1 min] x 50 cycles	117
		ICR2 R: ACCTCCACACCRAAA ACCCA	ICR2 UM: VIC- TGTGTGAGGATAGTGGTTGT-MGB		

The CpG position before sodium bisulfite DNA treatment are underlined.

Table 2. Mean MI and SD of CBS2 ICR1 and ICR2 calculated by ASMM RTQ-PCR and Southern blotting for all patients in the prospective study

	ICR1 11p15	ICR2 11p15
MI ASMMRTQ-PCR	50±10	42±14
MI Southern blotting	54±8	42±13

Table 3. Qualitative results for CBS2 ICR1 in the prospective study

ICR1 11p15		Southern blot conclusion		Total
		A	N	
ASMM RTQ-PCR Conclusion	A	8	0	8
	N	3	71	74
Total		11	71	82

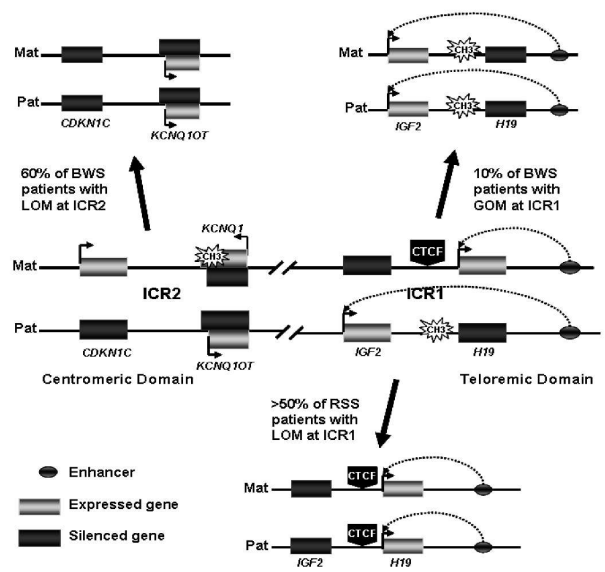
A= abnormal DNA methylation (LOM or GOM), N = normal DNA methylation

Table 4. Qualitative results for ICR2 in the prospective study

ICR2 11p15		Southern blotting conclusion		Total
		A	N	
ASMM RTQ-PCR Conclusion	A	10	2	12
	N	0	70	70
Total		10	72	82

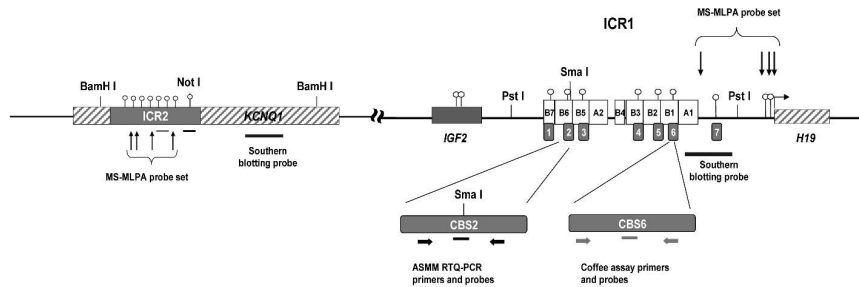
A= abnormal DNA methylation, N = normal DNA methylation

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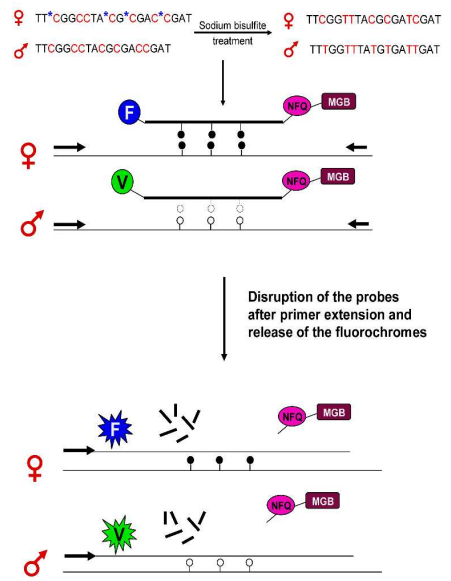
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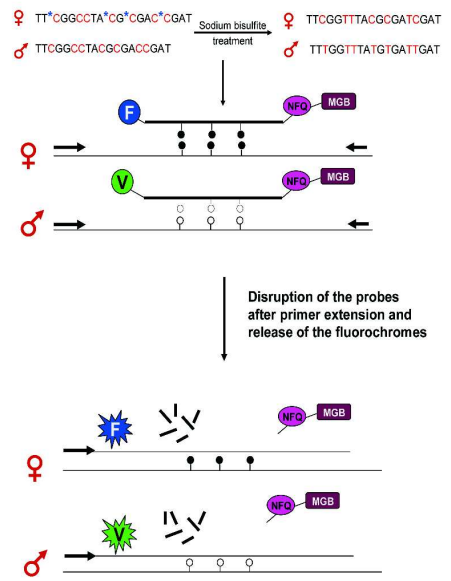
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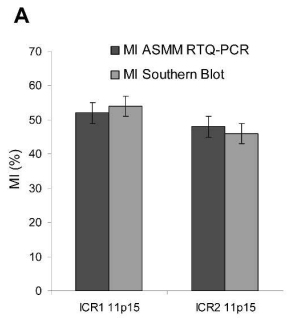
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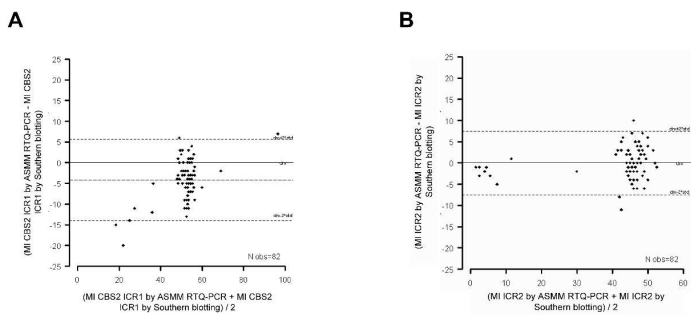
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Methods	ICR1 11p15	ICR2 11p15
MI ASMM RTQ-PCR (n = 64)	52±3	48±3
MI Southern Blot (n = 64)	54±3	46±3

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Review

Supplementary Materials

Figure and legends

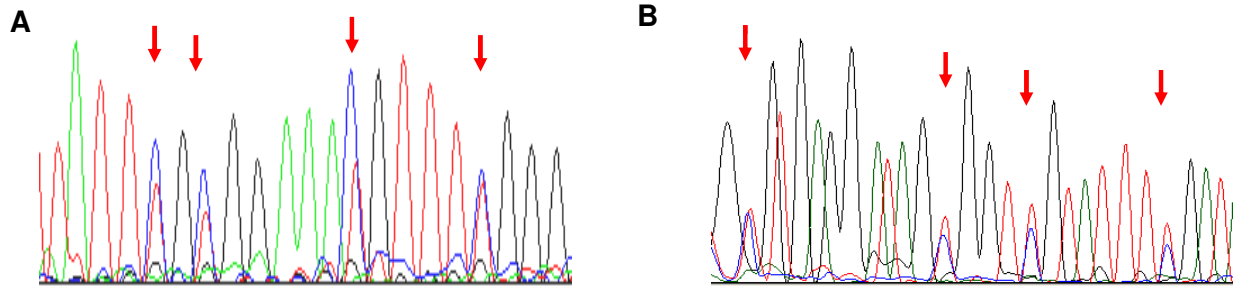


Figure S1: Electropherogram depicting the sequences of CBS2 ICR1 (A) and ICR2 (B). The C/T polymorphisms are indicated by the arrows.

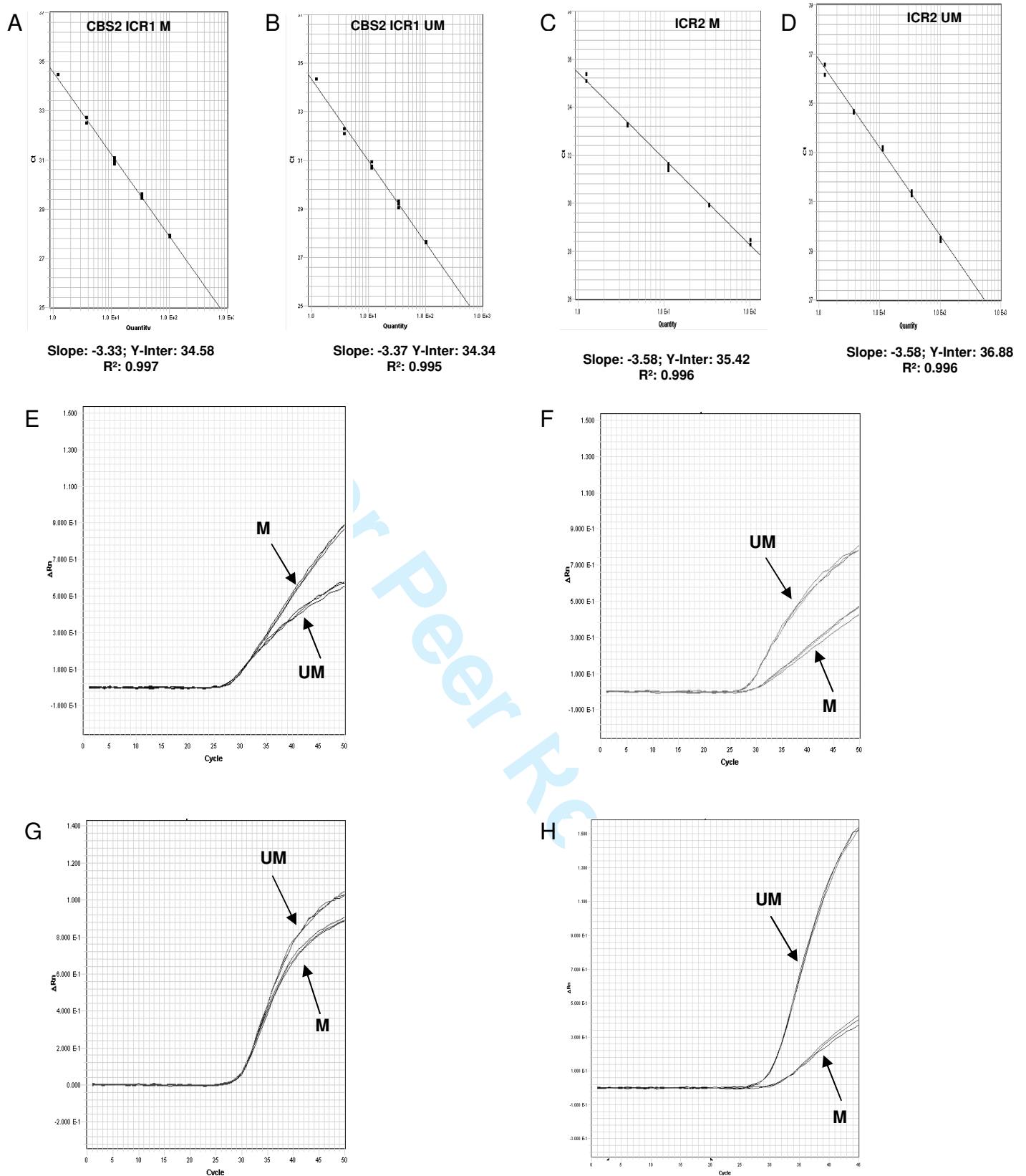


Figure S2: Standard curves for both unmethylated and methylated alleles of CBS2 ICR1 (A and B) and ICR2 (C and D). The slopes and the correlation coefficients are indicated under each standard curve. The amplification efficiency calculated from the slopes was up to 98% and 90%, for CBS2 ICR1 and ICR2, respectively. E-H show amplification curves for CBS2

ICR1 (E and F) and ICR2 (G and H) for a control subject (E and G) and an RSS patient (F) or BWS patient (H). The unmethylated and methylated curves overlap in the control situation, whereas in patients with loss of methylation at CBS2 ICR1 or ICR2, amplification of the unmethylated allele occurs several cycles earlier. M: methylated; UM: unmethylated.

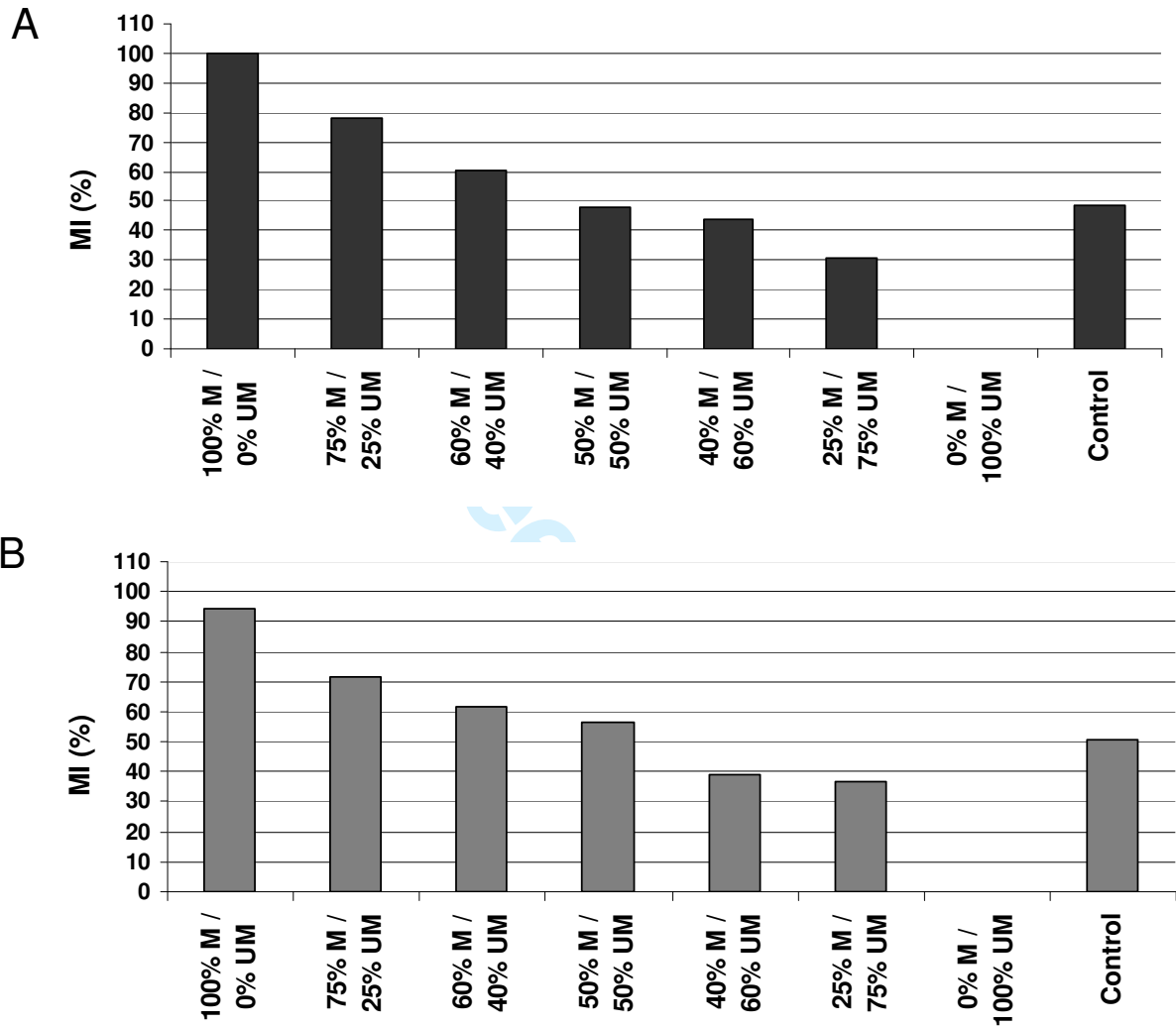


Figure S3: Concentration range of a mixture of EpiTech control DNAs (methylated and unmethylated) giving theoretical MIs of 100%, 75%, 60%, 50%, 40%, 25% and 0%, for both ICR1 (A) and ICR2 (B). The control DNA (pool of DNA from healthy subjects) was added as an internal control and showed a balance between the two alleles (50%). M: methylated and UM: unmethylated.

Figure S4:

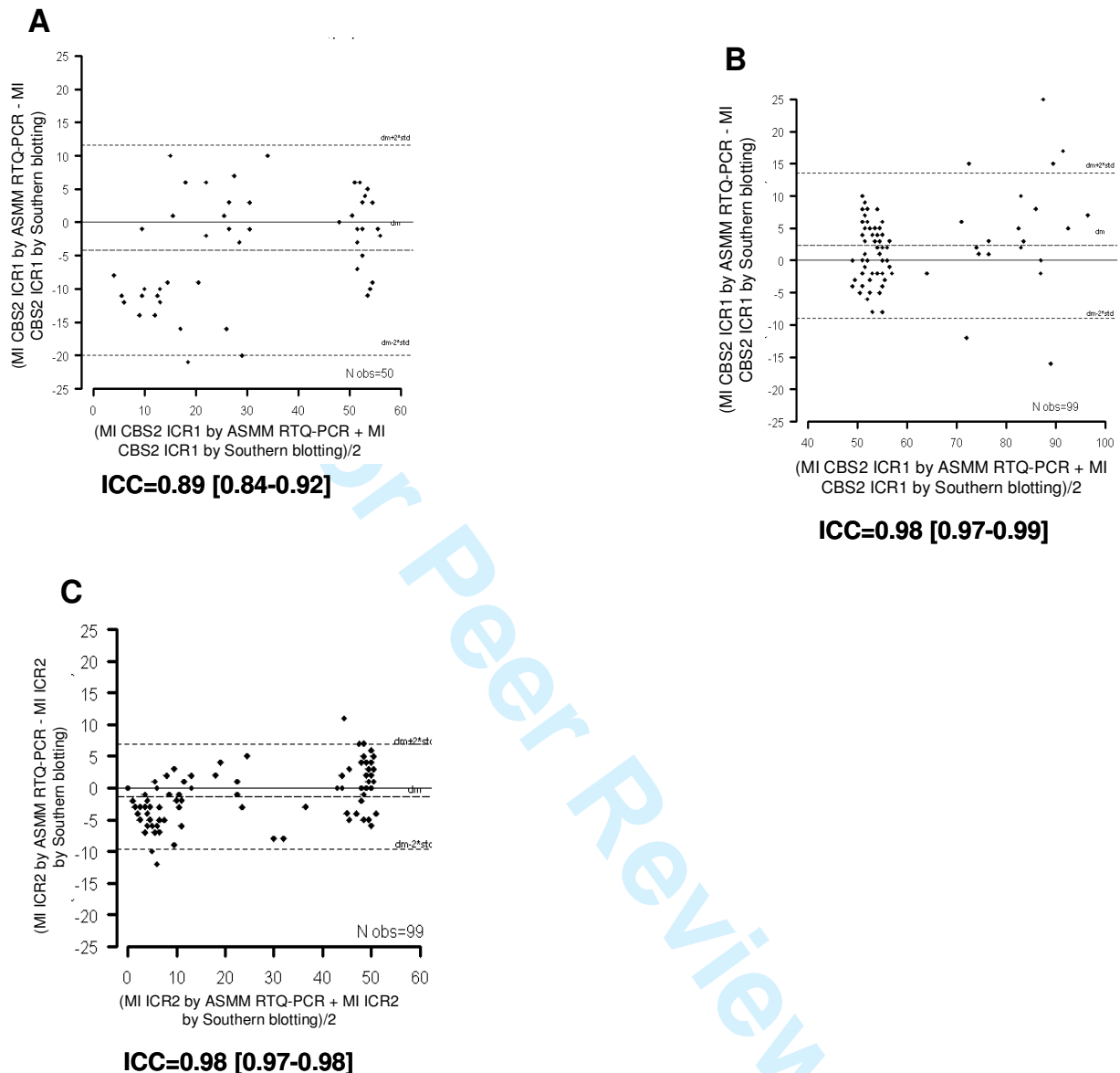


Figure S4: Bland and Altman's graphical representation of the intraclass correlation coefficient (ICC and its IC95% by bootstrap). ASMM RTQ-PCR and Southern blotting agreement in the retrospective RSS (A) and BWS (B and C) cohorts for CBS2 ICR1 and ICR2 (difference of MI measurements against their mean value). 'Limits of agreement' is defined by the mean difference (dm) and the standard deviation of the difference as $dm \pm SD$ for CBS2 ICR1 in both RSS (A) and BWS (B) cohorts and for ICR2 in the BWS cohort (C).

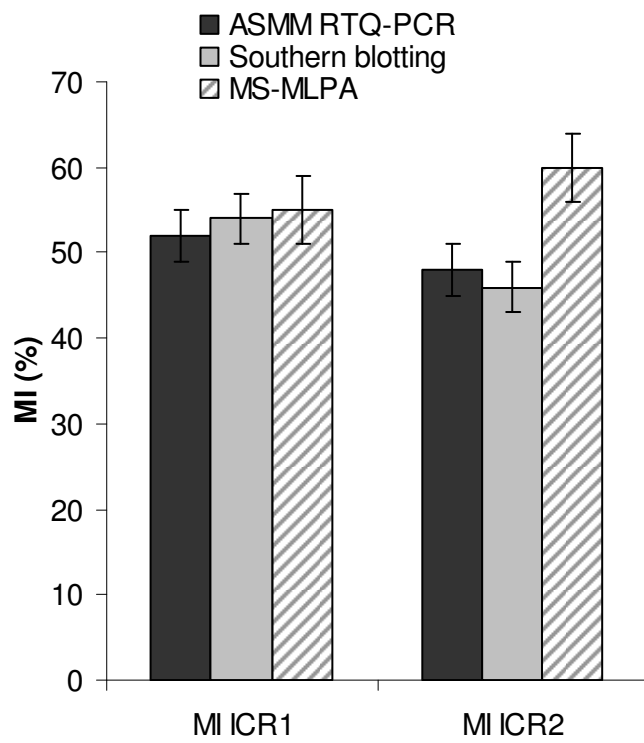


Figure S5: comparison between ICR1 and ICR2 MIs obtained with ASMM RTQ-PCR, Southern blotting and MS-MLPA.

Tables

Table S1: Qualitative results for CBS2 ICR1 for the RSS population in the retrospective study

ICR1 11p15	Southern blotting conclusion		Total	
	A	N		
ASMM	A	31	0	31
RTQ-PCR Conclusion	N	0	19	19
	Total	31	19	50

Kappa coefficient = 1.000

A= abnormal DNA methylation (LOM), N = normal DNA methylation

Table S2: Qualitative results for CBS2 ICR1 for the BWS population in the retrospective study

ICR1 11p15	Southern blotting conclusion		Total	
	A	N		
ASMM	A	21	0	21
RTQ-PCR Conclusion	N	3	75	78
	Total	24	75	99

Kappa coefficient = 0.9138

A= abnormal DNA methylation (LOM or GOM), N = normal DNA methylation

Table S3: Qualitative results for ICR2 for the BWS population in the retrospective study

ICR2 11p15	Southern blotting conclusion		Total	
	A	N		
ASMM	A	63	0	63
RTQ-PCR Conclusion	N	0	36	36
	Total	63	36	99

Kappa coefficient = 1.000

A= abnormal DNA methylation (LOM), N = normal DNA methylation

Table S4: Diagnostic established by ASMM RTQ-PCR, Southern blotting and MS-MLPA for a prospective population patients (n=69). The row highlighted in yellow represent patients with MS-MLPA discordant MI and those highlighted in green represent the discordant MI obtained with either ASMM RTQ-PCR or Southern blotting. The discordant MIs are in bleu.

Patient ID	ASMM RTQ-PCR				Southern Blotting				MS-MLPA			
	52±3 (46-58)		48±3 (42-54)		54±3 (48-60)		46±3 (40-53)		55±3 (49-61)		60±4 (52-68)	
	MI ICR1	DIAG	MI ICR2	DIAG	MI ICR1	DIAG	MI ICR2	DIAG	MI ICR1	DIAG	MI ICR2	DIAG
582	49	N	2	LOM	57	N	3	LOM	55	N	11	LOM
641	50	N	43	N	57	N	45	N	54	N	58	N
771	48	N	38	LOM	53	N	46	N	55	N	57	N
583	47	N	44	N	56	N	45	N	63	GOM	71	GOM
627	51	N	48	N	55	N	52	N	53	N	56	N
766	51	N	49	N	51	N	46	N	55	N	55	N
635	57	N	52	N	55	N	53	N	52	N	55	N
720	49	N	50	N	58	N	49	N	53	N	54	N
642	51	N	50	N	56	N	46	N	53	N	54	N
722	53	N	1	LOM	58	N	4	LOM	54	N	11	LOM
760	50	N	43	N	55	N	49	N	54	N	57	N
553	52	N	45	N	59	N	46	N	65	GOM	66	N
554	47	N	42	N	56	N	40	N	53	N	52	N
651	54	N	4	LOM	53	N	7	LOM	54	N	11	LOM
590	48	N	44	N	57	N	48	N	57	N	56	N
643	51	N	48	N	51	N	50	N	51	N	55	N
652	49	N	47	N	49	N	47	N	51	N	57	N
653	47	N	44	N	50	N	50	N	55	N	61	N
684	51	N	44	N	53	N	50	N	54	N	56	N
637	53	N	4	LOM	56	N	5	LOM	54	N	14	LOM
721	48	N	47	N	59	N	50	N	55	N	57	N
685	52	N	48	N	49	N	47	N	53	N	58	N
398	30	LOM	44	N	42	LOM	46	N	40	LOM	59	N
568	57	N	45	N	63	GOM	40	N	58	N	51	LOM
686	53	N	46	N	58	N	46	N	58	N	58	N
765	55	N	50	N	54	N	46	N	52	N	56	N
719	49	N	47	N	58	N	50	N	56	N	59	N
759	52	N	51	N	57	N	41	N	52	N	54	N
654	47	N	49	N	50	N	47	N	57	N	56	N
645	51	N	50	N	51	N	48	N	53	N	54	N
646	53	N	49	N	53	N	45	N	53	N	58	N
767	52	N	50	N	55	N	46	N	51	N	56	N
585	47	N	48	N	58	N	48	N	64	GOM	67	N
594	55	N	46	N	58	N	46	N	55	N	58	N
639	50	N	44	N	53	N	44	N	54	N	57	N
655	52	N	50	N	46	LOM	45	N	57	N	60	N
586	53	N	45	N	59	N	45	N	57	N	70	GOM
631	34	LOM	53	N	39	LOM	50	N	31	LOM	54	N

683	48	N	46	N	57	N	52	N	50	N	53	N
656	49	N	3	LOM	48	N	5	LOM	48	LOM	14	LOM
596	51	N	43	N	49	N	48	N	58	N	56	N
769	51	N	45	N	53	N	43	N	54	N	57	N
657	49	N	5	LOM	55	N	10	LOM	53	N	15	LOM
658	49	N	1	LOM	52	N	2	LOM	57	N	15	LOM
681	56	N	44	N	57	N	44	N	58	N	58	N
598	12	LOM	46	N	32	LOM	43	N	26	LOM	55	N
648	48	N	50	N	53	N	46	N	54	N	59	N
682	54	N	48	N	57	N	47	N	54	N	69	GOM
649	55	N	46	N	52	N	45	N	54	N	54	N
640	68	GOM	29	LOM	70	GOM	31	LOM	67	GOM	41	LOM
659	46	N	48	N	50	N	50	N	53	N	56	N
587	48	N	45	N	53	N	46	N	92	GOM	62	N
599	51	N	43	N	48	N	45	N	53	N	58	N
588	46	N	45	N	51	N	43	N	49	N	55	N
724	46	N	47	N	59	N	50	N	51	N	53	N
633	18	LOM	52	N	32	LOM	52	N	33	LOM	62	N
565	50	N	52	N	57	N	45	N	64	GOM	65	N
545	100	GOM	9	LOM	85	GOM	15	LOM	95	GOM	20	LOM
537	63	GOM	35	LOM	65	GOM	38	LOM	69	GOM	50	LOM
539	62	GOM	33	LOM	79	GOM	14	LOM	88	GOM	25	LOM
573	62	GOM	30	LOM	72	GOM	25	LOM	73	GOM	45	LOM
577	64	GOM	29	LOM	69	GOM	31	LOM	68	GOM	43	LOM
514	65	GOM	28	LOM	65	GOM	30	LOM	71	GOM	44	LOM
348	0	LOM	2	LOM	8	LOM	3	LOM	10	LOM	16	LOM
2160	71	GOM	24	LOM	74	GOM	24	LOM	81	GOM	33	LOM
2383	61	GOM	54	N	ND	ND	ND	ND	59	N	62	N
396	32	LOM	37	LOM	32	LOM	53	N	32	LOM	50	LOM
1199	67	GOM	48	N	83	GOM	50	N	81	GOM	67	N
369	31	LOM	ND	ND	24	LOM	53	N	56	N	59	N

LOM: Loss of methylation; GOM: gain of methylation; N: normal; ND: not determined; DIAG: diagnostic