CLINICAL GENETICS An International Journal of Genetics, Molecular and Personalized Medicine

Clin Genet 2011: 80: 83–88
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CLINICAL GENETICS
doi: 10.1111/j.1399-0004.2010.01514.x

Short Report

Silver-Russell patients showing a broad range of ICR1 and ICR2 hypomethylation in different tissues

Begemann M, Spengler S, Kanber D, Haake A, Baudis M, Leisten I, Binder G, Markus S, Rupprecht T, Segerer H, Fricke-Otto S, Mühlenberg R, Siebert R, Buiting K, Eggermann T. Silver-Russell patients showing a broad range of ICR1 and ICR2 hypomethylation in different tissues. Clin Genet 2011: 80: 83–88. © John Wiley & Sons A/S, 2010

In all known congenital imprinting disorders an association with aberrant methylation or mutations at specific loci was well established. However, several patients with transient neonatal diabetes mellitus (TNDM), Silver-Russell syndrome (SRS) and Beckwith-Wiedemann syndrome (BWS) exhibiting multilocus hypomethylation (MLH) have meanwhile been described. Whereas TNDM patients with MLH show clinical symptoms different from carriers with isolated 6q24 aberrations, MLH carriers diagnosed as BWS or SRS present only the syndrome-specific features. Interestingly, SRS and BWS patients with nearly identical MLH patterns in leukocytes have been identified. We now report on the molecular findings in DNA in three SRS patients with hypomethylation of both 11p15 imprinted control regions (ICRs) in leukocytes. One patient was a monozygotic (MZ) twin, another was a triplet. While the hypomethylation affected both oppositely imprinted 11p15 ICRs in leukocytes, in buccal swab DNA only the ICR1 hypomethylation was visible in two of our patients. In the non-affected MZ twin of one of these patients, aberrant methylation was also present in leukocytes but neither in buccal swab DNA nor in skin fibroblasts. Despite mutation screening of several factors involved in establishment and maintenance of methylation marks including *ZFP57*, *MBD3*, *DNMT1* and *DNMT3L* the molecular clue for the ICR1/ICR2 hypomethylation in our patients remained unclear. Furthermore, the reason for the development of the specific SRS phenotype is not obvious. In conclusion, our data reflect the broad range of epimutations in SRS and illustrate that an extensive molecular and clinical characterization of patients is necessary.

Conflict of interest

There is no conflict of interest for any of the contributing authors.

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Received 20 May 2010, revised and accepted for publication 19 July 2010

Silver-Russell syndrome (SRS, RSS; OMIM 180860) is a clinically and genetically heterogeneous disorder which is mainly characterized by severe intrauterine and postnatal growth restriction (<−2.0 SD). Typical clinical features include an asymmetry of body and limbs, relative macrocephaly, a triangular face and feeding difficulties in early childhood. In ~40% of patients disturbances

in the epigenetically regulated region 11p15 are detectable, in additional 10% a maternal uniparental disomy for chromosome 7 (upd(7)mat) can be diagnosed (for review (1)). In 11p15, two adjacent imprinted gene clusters are localized which are regulated by two separate imprinted control regions (ICRs). The telomeric ICR1 acts as a control element for *H19* and *IGF2* expression while expression of *KCNQ1* and *CDKN1C* is regulated by the centromeric ICR2. In >38% of patients with SRS, a hypomethylation of the ICR1 is detectable; further patients carry maternal duplications of the whole 11p15 chromosomal region or upd(11p15)mat (2). So far, only one SRS patient with a mutation restricted to the ICR2 has been observed (3).

Beckwith-Wiedemann syndrome (BWS; OMIM 130650) is genetically and at least in parts clinically opposite to SRS. This overgrowth syndrome has a variable phenotypic expression including macroglossia and abdominal wall defects as the most prominent signs. The epigenetic and genetic findings include an ICR2 hypomethylation in 50–60% of patients, an upd(11)pat in 20%, an ICR1 hypermethylation in 5%, paternal 11p15 duplications in 3%, and *CDKN1C* point mutations (for review (4)). Interestingly, there is an increased rate of monozygotic (MZ) twins in BWS which are nearly always phenotypically discordant (for review (5)).

In SRS and BWS as well as in the other known congenital imprinting disorders an association with aberrant methylation or mutations at specific loci was well established. However, several patients with transient neonatal diabetes mellitus (TNDM; OMIM 601410) exhibiting hypomethylation at further maternally imprinted loci in addition to the disease-specific locus in 6q24 have been reported (6, 7). In some of these patients, mutations in the *ZFP57* gene could be identified to be associated with the multilocus hypomethylation (MLH) (8). Recently, BWS and SRS patients with MLH in blood lymphocytes have also been reported; both paternally and maternally imprinted loci were affected in leukocytes of the probands (9–13).

We now report on our molecular findings in DNA from three SRS patients with hypomethylation of both ICRs in 11p15. The patients were initially referred for routine SRS diagnostic testing.

Genomic DNA was isolated from peripheral blood leukocytes from the patients, their sibs and their parents by a simple salting out procedure. Buccal swab DNA from both sides of the mouth was obtained using the innuPREP Swab DNA Kit (analytikjena, Jena/Germany). In case of patient 3 and her MZ twin, DNA from skin biopsies was available.

Routine screening for 11p15 imbalances and epimutations was performed by methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) using a commercially available kit (SALSA ME030BWS/RSS, MRC Holland, Amsterdam/NL). Methylation analyses for other imprinted loci were performed by MS-MLPA for the *SNRPN* and the *NDN* loci using a commercial kit (ME028-B1, MRC Holland) or locus-specific methylation-specific (MS) polymerase chain reaction (PCR) approaches. For these MS-PCRs, genomic DNA was bisulfite treated – according to the protocol recently published by Kanber et al. (14) or by using the EZ DNA Methylation Gold Kit from Zymo Research (Orange, CA). Locus-specific MS-PCRs were performed for *ARH1* (1p31.3; D. Kanber, personal communication), *PLAGL1* (6q24) (15), *GRB10/MEG1* (7p12) (8), *MEST/PEG1* (7q32) (16), *RB1* (13q14) (17), IG-DMR (14q32) (18, 19), *PEG3* (19q13) (7), *NESPAS*, *NESP* and *GNAS* (20q13) (8, 17). In addition, the methylation levels of the ICR1/ICR2 in 11p15 and of several CpGs of the differentially methylated regions (DMRs) of the imprinted genes *PLAGL1*, *GRB10*, *MEST*, *SNRPN*, *MEG3* and *PEG3* were determined by bisulfite pyrosequencing. Bisulfite pyrosequencing of the *PLAGL1* locus has been described recently (20). For the DMRs of the other loci, primers were designed using PyroMark Assay design 2.0 (primer sequences are available on request). Bisulfite pyrosequencing was performed as recently described (21).

MS-MLPA for 11p15 (epi)mutations revealed both ICR1 and ICR2 hypomethylation in peripheral blood lymphocytes of three patients referred as SRS (Fig. 1). In patients 1 and 2, the loss of methylation (LOM) at both loci was obvious while in patient 3 the methylation indices (MIs) for both loci revealed low level demethylation. The data were confirmed by pyrosequencing approaches.

We then analyzed genomic DNA from buccal swabs from the left and the right buccal side by MS-MLPA in the three patients. In all patients, hypomethylation of the ICR1 was confirmed but the ICR2 MIs were in the normal range in patients 1 and 3 (Fig. 1). Only in patient 2 ICR2 hypomethylation was confirmed. Strong differences in the range of hypomethylation were observed between both sides of the oral cavity, reflecting the high degree of variation of epigenetic mosaicism in an individual. DNA from skin biopsy of patient 3 showed the same methylation pattern as found in blood, i.e. a low level mosaicism for both ICR1 and ICR2 hypomethylation.

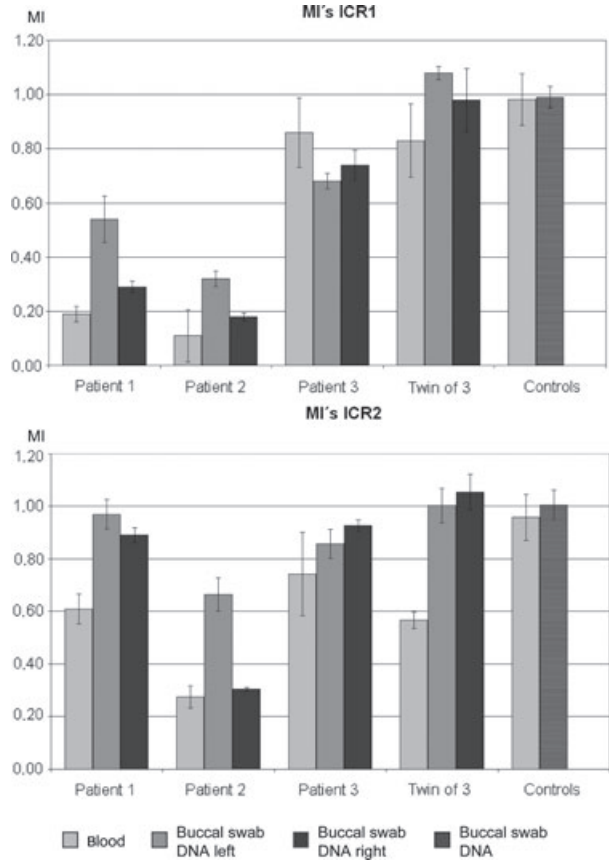

Fig. 1. Methylation indices (MIs) obtained by methylation-specific multiplex ligation-dependent probe amplification for the ICR1 and ICR2 loci in 11p15 in DNA obtained from blood and buccal swabs. In the three patients and the healthy monozygotic twin of patient 3, DNA from blood and from the left and right side of the oral cavity were tested, in case of the buccal swab DNA of the controls left and right were not differentiated.

Lymphocyte DNA of the three patients was also checked for further epimutations at other imprinted loci: in patients 1 and 3 we could not detect any further epimutation. In patient 2 hypomethylation at the *MEST/PEG1* locus was observed in both lymphocytes and buccal swab DNA. *MEST/PEG1* is believed to be involved in fetal growth, although its precise function is unknown. In mice, disruption of the *Mest/Peg1* gene causes embryonic growth retardation when paternally transmitted (22). However, since *MEST/PEG1* hypomethylation functionally corresponds to a upd(7)pat and this aberration is not associated with an imprinting disorder we assume that a phenotypic consequence of this epimutation is not obvious.

Interestingly, the healthy MZ twin sister of patient 3 carried a more severe hypomethylation of both ICRs in 11p15 in leukocytes than her affected sister (Fig. 1) but in DNA from buccal smear and skin fibroblasts, respectively, the methylation pattern was normal. This observation is consistent with recently published data from MZ twins discordant for BWS: in buccal swab DNA of BWS MZ twins aberrant methylation was detectable only in the affected twin whereas both twins showed similar abnormal methylation patterns in blood (5). The restriction of the ICR1/ICR2 hypomethylation to blood in the healthy twin of patient 3 agrees with the idea of a possible fetal sharing of hematopoietic cells circulation (23) in MZ twins because 70% of all MZ monochorionic twins share blood through vascular connections (24). It is also consistent with another hypothesis for the similar epimutation patterns in discordant MZ twins which comprises the migration of blood cell precursor cells from the yolk sac (5).

Recently, hypomethylation of both ICRs in 11p15 has also been described in BWS (5, 11). Thus it can be asked why the same hypomethylation pattern is associated with both SRS and BWS as opposite clinical pictures. In two of our patients the ICR1 hypomethylation was the only epimutation which was present in the DNA from different tissues while the ICR2 LOM was not. This finding is supported by the normal imprinting status of these regions in the second investigated tissue of the MZ twin of patient 3 carrying an ICR1 and ICR2 hypomethylation in lymphocytes. However, the epimutation mosaicism in our third patient was extremely broad. Azzi et al. (11) recently suggested that epigenetic mosaicism may vary between different tissues and tissue-specific distribution of this mosaicism might explain the clinical expression of either SRS or BWS. In addition, the date of disturbed methylation in ontogenesis has to be considered and has probably a great impact on the clinical course of the disease.

Clinically, our three patients do not obviously differ from SRS patients with isolated ICR1 hypomethylation (Table 1) (Fig. 2). This observation is consistent with that from other studies on SRS and BWS patients with MLH exhibiting the typical clinical courses but is different from TNDM where the phenotype of MLH carriers is altered in comparison to TNDM patients with isolated 6q24 hypomethylation (7). However, an influence of the ICR2 hypomethylation on the phenotype cannot be excluded: two of our carriers (patient 1 and the twin sib of patient 3) of ICR1 and ICR2 LOM showed an umbilical hernia, which is one of the key features of BWS.

The loss of paternal as well as of maternal methylation marks in our three patients with SRS can probably not be attributed to a deficient acquisition of methylation during gametogenesis but is consistent with an incorrect maintenance of

Table 1. Phenotype of the three ICR2 hypomethylation carriers in comparison to SRS patients with isolated ICR1 hypomethylation

Clinical features	Patient 1	Patient 2	Patient 3	MZ twin of patient 3	Isolated H19 hypomethylation carriers[a]	Idiopathic SRS[a]
Sex	Female	Male	Female			
ART	No	IvF	No[b]			
Twins/triplets	No	Triplet, not MZ	MZ twin			
Age at last examination and blood drawing	17 years	15 years	9 months			
Gestational week	At term	36	31 + 5			
Family history	Unremarkable	Unremarkable	Unremarkable			
Growth parameters[c]						$n = 129$
Birth weight (SD)	2240 g (−2.92 SD)	980 g (−4.22 SD)	995 g (−1.88 SD)	2280 g (2.03 SD)	−3.55 SD ($n = 58$)	−3.1
Birth length (SD)	46 cm (−2.59 SD)	35 cm (−5.42 SD)	35 cm (−1.74 SD)	43 cm (0.54 SD)	−4.38 SD ($n = 57$)	−4.1
Birth OFC (SD)	34.0 cm (−0.69 SD)	30.5 cm (−2.06 SD)	28 cm (−0.45 SD)	36.5 cm (3.55 SD)	−1.35 SD ($n = 54$)	−1.5
Postnatal growth retardation (<−2 SD)	(No)[d]	Yes	Yes	No		
Clinical features						$n = 388$
Relative macrocephaly	Yes	Yes	Yes		91% ($n = 59$)	68.4%
Muscular hypotonia	+	+	(+)		−	45%
Asymmetry	+	+	+		77% ($n = 57$)	53.1%
Clinodactyly	+	+	+		78% ($n = 40$)	69.9%
Squeaky voice	+	+				
Neonatal feeding difficulties	+	−	+			
Developmental delay	No (high school)	No (high school)	Too young	Too young	20.5% ($n = 31$)	32.2%
Craniofacial features						
Triangular face	+	+	+		76% ($n = 59$)	78.4%
Prominent forehead	+	+	+		88% ($n = 34$)	72.4%
Downturned corners of the mouth	+	+	+		55% ($n = 9$)	57.3%
Micrognathia	+	+	+		55% ($n = 9$)	44%
Ear anomalies	+	+	+			40.3%
Irregular spacing of teeth	+	+	Too young		0% ($n = 7$)	28%
Other features	Umbilical hernia, Scoliosis	Small tongue, Scoliosis		Umbilical hernia		

ART, assisted reproductive technology; GH, growth hormone; ICR, imprinted control regions; IVF, in-vitro fertilization; MZ, monozygotic; SRS, Silver-Russell syndrome.

[a]Reviewed by Kotzot (25).

[b]The older sister was born after IvF but the gemini were products of a normal conception.

[c]SD scores were based on singleton data because data from multiples were not available.

[d]After GH therapy: −1.63 SD.

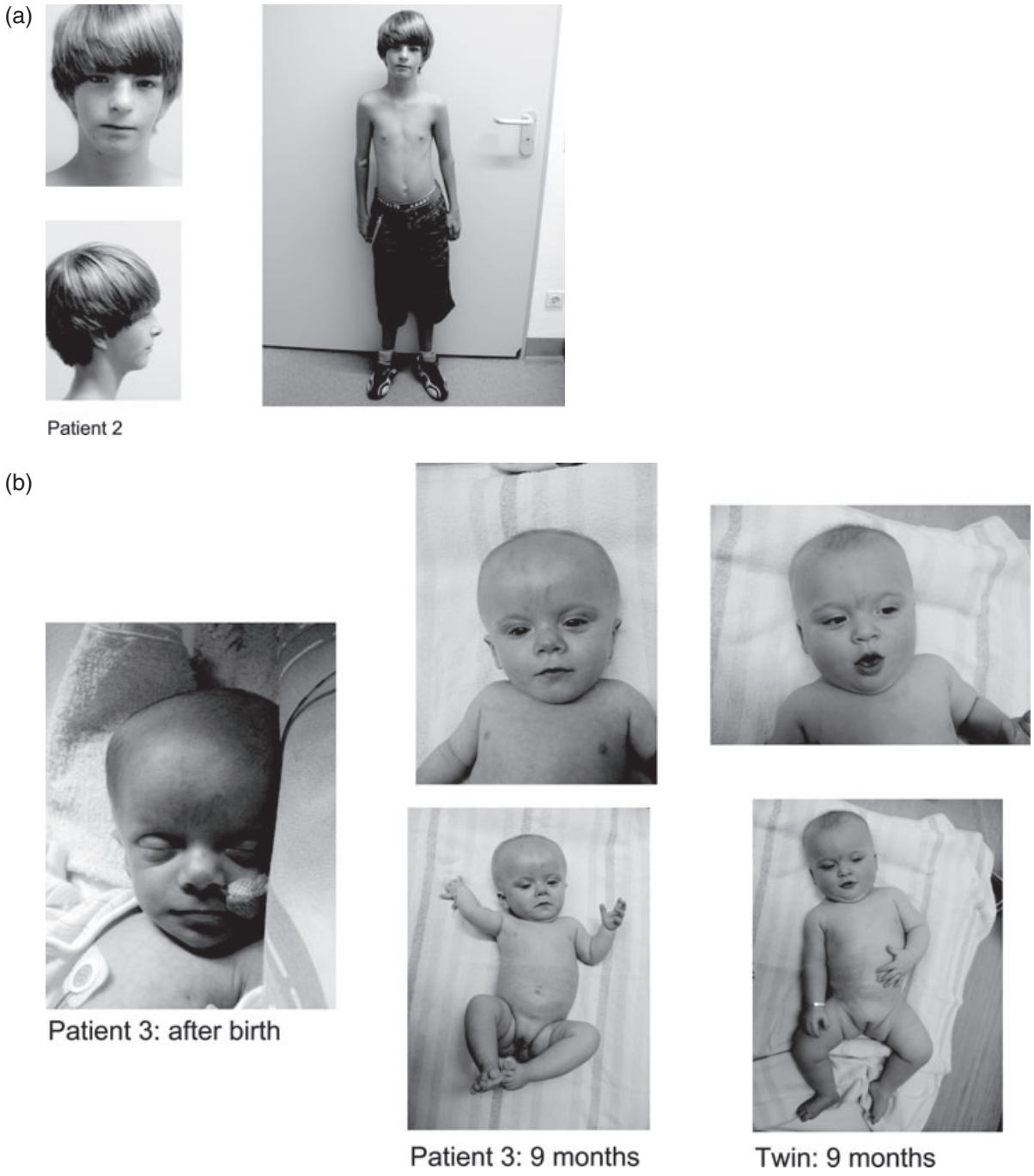

Fig. 2. Clinical presentation of (**a**) patient 2 at the age of 15 years; (**b**) patient 3 after birth (left), the patient (middle) and her twin sister (right) at the age of 9 months.

methylation after fertilization. To further investigate the underlying defect of the ICR1/ICR2 hypomethylation we screened coding regions of the *ZFP57*, *DNMT3L*, *DNMT1* and *MBD3* genes for mutations in the three patients. All these factors have been functionally associated with establishment and maintenance of methylation marks in human, but we failed to detect any pathogenic mutation in the coding regions of these genes. We furthermore excluded submicroscopic imbalances by SNP array typing (SNP 6.0 array, Affymetrix, High Wycombe, UK).

Our results and those from other groups show that ICR1/ICR2 hypomethylation in SRS is not rare (11, 13): among our 77 ICR1 hypomethylation carriers identified in routine diagnostics, three showed the additional ICR2 hypomethylation. This rate of 3.8% is similar to that reported by Azzi

et al. (11) of 4.05% (3 out of 74 ICR1 positive patients). Molecular genetic testing for SRS should therefore not be restricted to the ICR1 in 11p15 but should comprise further loci. As our data from different tissues shows, the extent of mosaicism for epimutations in the same individual can be broad. In case of ICR1/ICR2 hypomethylation the phenotype is probably caused by the predominant epimutation in that tissue and that ontogenetic stage which is relevant for the course of the disease.

Acknowledgements

We thank all families participating in this study. The project was supported by BMBF and the Else Kröner Fresenius-Stiftung.

References

1. Eggermann T, Schönherr N, Eggermann K et al. Use of multiplex ligation-dependent probe amplification increases the detection rate for 11p15 epigenetic alterations in Silver-Russell syndrome. Clin Genet 2008: 73 (1): 79–84.
2. Bullman H, Lever M, Robinson DO, Mackay DJG, Holder SE, Wakeling EL. Mosaic maternal uniparental disomy of chromosome 11 in a patient with Silver-Russell syndrome. J Med Genet 2008: 45: 396–399.
3. Schönherr N, Meyer E, Roos A, Schmidt A, Wollmann HA, Eggermann T. The centromeric 11p15 imprinting centre is also involved in Silver-Russell syndrome. J Med Genet 2007: 44: 59–63.
4. Cooper WN, Luharia A, Evans GA et al. Molecular subtypes and phenotypic expression of Beckwith-Wiedemann syndrome. Eur J Hum Genet 2005: 13: 1025–1032.
5. Bliek J, Alders M, Maas SM et al. Lessons from BWS twins: complex maternal and paternal hypomethylation and a common source of haematopoietic stem cells. Eur J Hum Genet 2009: 17: 1625–1634.
6. Arima T, Kamikihara T, Hayashida T et al. ZAC, LIT1 (KCNQ1OT1) and p57KIP2 (CDKN1C) are in an imprinted gene network that may play a role in Beckwith-Wiedemann syndrome. Nucleic Acids Res 2005: 33: 2650–2660.
7. Mackay DJG, Boonen SE, Clayton-Smith J et al. A maternal hypomethylation syndrome presenting as transient neonatal diabetes mellitus. Hum Genet 2006: 120: 262–269.
8. Mackay DJG, Callaway JLA, Marks SM et al. Hypomethylation of multiple imprinted loci in individuals with transient neonatal diabetes is associated with mutations in ZFP57. Nat Genet 2008: 40: 949–951.
9. Rossignol S, Steunou V, Chalas C et al. The epigenetic imprinting defect of patients with Beckwith-Wiedemann syndrome born after assisted reproductive technology is not restricted to the 11p15 region. J Med Genet 2006: 43: 902–907.
10. Bliek J, Verde G, Callaway J et al. Hypomethylation at multiple maternally methylated imprinted regions including PLAGL1 and GNAS loci in Beckwith-Wiedemann syndrome. Eur J Hum Genet 2009: 17: 611–619.
11. Azzi S, Rossignol S, Steunou V et al. Multilocus methylation analysis in a large cohort of 11p15-related foetal growth disorders (Russell Silver and Beckwith Wiedemann syndromes) reveals simultaneous loss of methylation at paternal and maternal imprinted loci. Hum Mol Genet 2009: 18: 4724–4733.
12. Horike S, Ferreira JCP, Meguro-Horike M et al. Screening of DNA methylation at the H19 promoter or the distal region of its ICR1 ensures efficient detection of chromosome 11p15 epimutations in Russell-Silver syndrome. Am J Med Genet A 2009: 149A: 2415–2423.
13. Turner CLS, Mackay DM, Callaway JLA et al. Methylation analysis of 79 patients with growth restriction reveals novel patterns of methylation change at imprinted loci. Eur J Med Genet 2010: 17: 648–655.
14. Kanber D, Buiting K, Zeschnigk M, Ludwig M, Horsthemke B. Low frequency of imprinting defects in ICSI children born small for gestational age. Eur J Hum Genet 2009: 17: 22–29.
15. Mackay DJG, Temple IK, Shield JPH, Robinson DO. Bisulphite sequencing of the transient neonatal diabetes mellitus DMR facilitates a novel diagnostic test but reveals no methylation anomalies in patients of unknown aetiology. Hum Genet 2005: 116: 255–261.
16. Moore MW, Dietz LG, Tirtorahardjo B, Cotter PD. A multiplex methylation PCR assay for identification of uniparental disomy of chromosome 7. Hum Mutat 2003: 21: 645–648.
17. Kanber D, Berulava T, Ammerpohl O et al. The human retinoblastoma gene is imprinted. PLoS Genet 2009: 5: e1000790.
18. Dietz LG, Wylie AA, Rauen KA, Murphy S, Jirtle R, Cotter P. Exclusion of maternal uniparental disomy of chromosome 14 in patients referred for Prader-Willi syndrome using a multiplex methylation polymerase chain reaction assay. J Med Genet 2003: 40: e46.
19. Temple IK, Shrubb V, Lever M, Bullman H, Mackay DJG. Isolated imprinting mutation of the DLK1/GTL2 locus associated with a clinical presentation of maternal uniparental disomy of chromosome 14. J Med Genet 2007: 44: 637–640.
20. Martin-Subero JI, Bibikova M, Mackay D et al. Microarray-based DNA methylation analysis of imprinted loci in a patient with transient neonatal diabetes mellitus. Am J Med Genet A 2008: 146A: 3227–3229.
21. Martín-Subero JI, Kreuz M, Bibikova M et al. New insights into the biology and origin of mature aggressive B-cell lymphomas by combined epigenomic, genomic, and transcriptional profiling. Blood 2009: 113: 2488–2497.
22. Lefebvre L, Viville S, Barton SC, Ishino F, Keverne EB, Surani MA. Abnormal maternal behaviour and growth retardation associated with loss of the imprinted gene Mest. Nat Genet 1998: 20: 163–169.
23. Weksberg R, Shuman C, Caluseriu O et al. Discordant KCNQ 1OT1 imprinting in sets of monozygotic twins discordant for Beckwith-Wiedemann syndrome. Hum Mol Genet 2002: 11: 1317–1325.
24. Hall JG. Twinning. Lancet 2003: 362 (9385): 735–743.
25. Kotzot D. Maternal uniparental disomy 7 and Silver-Russell syndrome – clinical update and comparison with other subgroups. Eur J Med Genet 2008: 51: 444–451.