The significance of molecular studies in the long-term follow-up of children with Beckwith-Wiedemann syndrome

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Beckwith–Wiedemann syndrome (BWS) is a congenital disorder of imprinting caused by epimutations and mutations affecting two imprinted loci on chromosome 11p15. Its clinical features are heterogeneous, including macrosomia, macroglossia, hemihyperplasia, abdominal wall defects, neonatal hypoglycemia, and increased risk of embryonal tumors such as Wilms tumor, adrenocortical carcinoma, hepatoblastoma, and neuroblastoma. The molecular and clinical heterogeneity of BWS makes the diagnosis challenging, but essential, since different etiologies of BWS have different clinical prognoses - most crucially, patients with gain of maternal methylation at imprinting control region type 1 (ICR1) are at significant risk of Wilms tumor or hepatoblastoma.

We present three cases of BWS with different symptomatology and two different molecular diagnoses. The authors emphasize the importance of molecular studies in the long-term follow-up of children with BWS, including refinement of phenotype-genotype correlation and its connection with optimal management and tumor surveillance.

Key words: Beckwith–Wiedemann syndrome (BWS), methylation, imprinting disorder, chromosome 11p15.

Genomic imprinting is a genetic phenomenon by which the expression of particular genes is regulated in a parent-of-origin manner. Imprinted alleles are "turned off" so that genes are expressed only from the non-imprinted alleles inherited from the mother (e.g. *H19* or *CDKN1C*) or the father (e.g. insulin-like growth factor (*IGF 2*)[1]. This process involves methylation and histone modifications in order to achieve monoallelic gene expression without altering the genetic sequence. Epigenetic marks are established in the germline and are present in all somatic cells[2].

Beckwith-Wiedemann syndrome (BWS, OMIM 13650) is a congenital disorder of imprinting associated with overgrowth and tumor predisposition[3]. Its prevalence is estimated at approximately 1 in every 13,700 live births, manifesting equally in males and females[4]. The clinical picture is highly variable with generalized hyperplasia, neonatal hypoglycemia, abdominal wall defects, macroglossia, and posterior helical ear pits as the main clinical features[5-7]. Children with BWS have an increased susceptibility (7.5%) to tumors such as Wilms tumor, adrenocortical carcinoma, hepatoblastoma, and neuroblastoma[8].

Beckwith-Wiedemann syndrome (BWS) is also heterogeneous in molecular etiology, being caused by genetic and epigenetic defects of two closely-apposed clusters of imprinted genes on chromosome 11p15.5. The BWS critical region

includes two domains: imprinting control region 1 (ICR1) regulates the expression of *IGF2* and *H19* in domain 1, while imprinting control region 2 (ICR2) regulates the expression of *CDKN1C* and *KCNQ1OT1* genes in domain 2[4,9]. Approximately 55% of patients show loss of maternal methylation at ICR2, while ~5% have gain of methylation on the maternal allele of ICR1; chromosomal and copy number defects are present in ~20% of cases and *CDKN1C* mutations in 5%; no cause is known in ~15%[10-12]. While most BWS is apparently sporadic, transmission is observed in ~15-20% of cases[13]. Gain of maternal methylation at ICR1 is the defect with the highest risk of neoplasm[14].

The molecular heterogeneity and the variations in presentation and prognosis in BWS indicate that an accurate molecular diagnosis is a prerequisite for long-term management of affected children, especially with reference to oncology prophylaxis. We present three cases of BWS with different symptomatology and two different molecular diagnoses.

Molecular Methods

Methylation analysis of ICR1 and ICR2 differentially-methylated regions was performed by methylation-specific polymerase chain reaction (PCR) and pyrosequencing as described[15]. Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) was performed using kit ME-030 v1 (MRC-Holland) according to the manufacturer's instructions. Microsatellite analysis of chr11p15 was performed by standard methods.

Case Reports

Case 1

A male infant with hypoglycemia, macroglossia and hemihyperplasia was born to young, healthy and unrelated parents after 40 weeks of gestation, with birth weight 3980 g, birth length 60 cm, head circumference 36 cm, and Apgar score 9/10 points. The physical examination revealed enlargement of the tongue causing respiratory difficulties on the 2nd day of life (Fig. 1), left lower limb hemihyperplasia, facial nevus flammeus, and jaundice. In the laboratory assessment, hypoglycemia (lowest glucose level 1.6 mmol/L), hyperbilirubinemia (total bilirubin 333.5 μmol/L, conjugated bilirubin 11.73 μmol/L) and elevated level of serum alpha-fetoprotein (AFP) (19300 IU/ml) were

detected. The patient was intensively treated with glucose infusion and frequent feedings to maintain normoglycemia. At the age of nine months, surgical reduction of macroglossia was performed. At 10 months, the child's development was appropriate for his age, AFP levels remained within the reference range, and no pathological changes were detected by abdominal ultrasound. Molecular DNA testing revealed borderline loss of methylation (LOM) at *KCNQT1OT1* (ICR2), partial gain of methylation (GOM) at *H19* (ICR1) and *IGF2P0*, and a methylation pattern consistent with mosaic paternal uniparental disomy (UPD) of 11p15.5 - findings typical for BWS; this was confirmed by microsatellite analysis. Due to the increased risk of tumor development and the history of neonatal hypoglycemia, the patient remains under systematic follow-up including evaluation of psychomotor development, ultrasound examination of the abdomen and monitoring of AFP level.

Case 2

This male child of young, healthy and unrelated parents was delivered naturally in the 39th week of pregnancy with birth weight 3950 g, birth length 57 cm, head circumference 37 cm, and Apgar score 10/10/10 points. Neonatal glycemia data were not recorded.

At the age of 10 months, transfontanel ultrasound examination revealed a hyperechogenic lesion (hemangioma–like) in the left lateral ventricle area. The child was admitted with good clinical status, age-appropriate psychomotor and physical development (weight SD 0; height SD 0.5; head circumference SD -0.6), left-sided hemihyperplasia, nevus flammeus of the forehead and philtrum, and posterior helical ear pits. Concentration of AFP was slightly increased (12.17 IU/ml). No abnormalities of the heart or abdomen were observed by diagnostic imaging. Magnetic resonance imaging of the head revealed dilatation of the subarachnoidal space in the frontal, parietal and temporal lobe areas and choroid plexus cysts in posterior horns of lateral ventricles; the patient was referred for further clinical observation. On genetic examination, partial LOM at the *KCNQ1OT1* (ICR2) gene was detected, which, together with the clinical presentation, was consistent with the diagnosis of BWS. Additionally, uncommon for BWS,

changes were observed at the following loci: *IGF2R* (partial loss), *GRB10* (total loss), *MEST* (partial loss), and *H19* (partial loss). The patient remains under regular pediatric surveillance due to increased risk of neoplasm.

Case 3

The male child of healthy, unrelated parents (mother 37 years, father 36 years) was delivered at 42 weeks of gestation with birth weight 4000 g, birth length 56 cm, head circumference 36.5 cm, and Apgar score 10/10/10 points, with left-sided macroglossia accompanied by sublingual lesion suspected as hemangioma, and no history of hypoglycemia. At the age of three months, the sublingual lesion was removed, but histopathological examination of salivary gland tissue showed no morphological abnormalities.

At the age of 17 months, the child had good general status, with age-appropriate psychomotor and physical development (weight SD 2.5; height SD 0; head circumference SD 0.7), left-sided hemihyperplasia, macroglossia, tongue asymmetry with enlargement of the left side, and nevus flammeus of the forehead.

Concentration of AFP was normal, abdominal ultrasound revealed no abnormalities, and cardiac ultrasound revealed partial thickening of the intraventricular septum.

Molecular testing revealed typical findings for BWS DNA - subtle hypermethylation at *H19* (ICR1) and *IGF2-DMR0*, hypomethylation at *KCNQT1OT1* (ICR2), and mosaic paternal UPD of 11p15.5; this was confirmed by microsatellite analysis. The patient remains under regular pediatric follow-up due to increased risk of neoplasm.

The clinical features of the three patients with genetic confirmation of BWS are presented in Table I.

Discussion

Loss of imprinting (LOI) through loss (LOM) or gain (GOM) of methylation is involved in many human disorders and neoplasms. The imprinted 11p15 region is crucial for the control of fetal growth and contains two imprinted domains[16]. LOI at this locus is implicated in two clinically opposite disorders: BWS with fetal overgrowth associated with enhanced tumor risk and Silver-Russell syndrome (SRS) with intrauterine and postnatal growth restriction[16].

A comprehensive molecular diagnosis of BWS and SRS requires both epigenetic and genetic testing to detect the diverse DNA methylation and copy number changes that can cause these disorders. MS-MLPA is a widely used diagnostic tool, which can detect copy number changes and DNA methylation changes including UPD; however, it has limited sensitivity to very low-level mosaic epimutations and UPD. Translocations/inversions of *KCNQ1* are not usually detectable[17].

Recent genotype-phenotype correlation data highlight the varying risk of neoplasm of different (epi)mutations[18]. Several clinical studies suggest an increased risk of BWS associated with assisted reproductive technology (ART)[19,20].

In all of our patients, the clinical diagnosis of BWS was confirmed by molecular studies. The clinical presentations of the probands were heterogeneous: in all cases, hemihyperplasia and nevus flammeus were observed, while other findings typical for BWS, including macroglossia, macrosomia and visceromegaly, were not observed consistently. Severe neonatal hypoglycemia was present only in Case 1; no abdominal wall defects were observed, and no family history for BWS was present in any case.

In Cases 1 and 3, methylation-specific PCR (MSP) showed hypomethylation at *KCNQ1OT1* (ICR2) and hypermethylation at *H19* (ICR1); this pattern was consistent with mosaic UPD11pat, which was confirmed in both cases by microsatellite analysis. Paternal UPD is observed in approximately 20% of patients with BWS[10]. They present somatic mosaicism; therefore, paternal UPD may be found only in some tissues, e.g., fibroblasts or renal tissue. Clinical features in this subgroup of BWS include hemihyperplasia and hyperinsulinemic hypoglycemia (with somatic mosaicism for 11p15 limited to the pancreas), which were also present in our case[21]. Individuals with 11p15 UPD and *H19* hypermethylation carry very high (10% up to about age 10) risk of tumor development, particularly Wilms tumor[13].

In Case 2, MSP showed partial LOM of *KCNQ1OT1* (ICR2). This patient carries lower tumor risk (in some individuals hepatoblastomas, rhabdomyosarcomas and gonadoblastomas were

Table I. Comparison of Clinical Features in Three Patients with Genetic Confirmation of BWS

Feature	Case 1	Case 2	Case 3
Abdominal wall defect	No	No	No
Macroglossia	Yes	No	Yes
Macrosomia	+/-	-	+/-
Ear changes	No	Yes	No
Visceromegaly	No	No	No
Hemihyperplasia	Yes	Yes	Yes
Family history for BWS	No	No	No
Nevus flammeus	Yes	Yes	Yes
Cardiac abnormalities	No	No	Yes
Neonatal hypoglycemia	Yes	No	No
H19 methylation ratio (mean±SD)	0.65±0.06	0.36±0.03	0.69±0.07
KCNQ1OT1 methylation ratio (mean±SD)	0.40±0.06	0.33±0.04	0.42±0.05

Methylation ratio calculated as mean value of indices of methylation probes in MLPA kit ME030-B2, as in Scott et al. 2008. Control values were H19: 0.52±0.04, KCNQ1OT1 0.57±0.05.

described, but not Wilms tumor)[14]. Patients with BWS due to LOM can also present with hemihypertrophy and omphalocele. Positive family history at ICR2 defects are observed rarely[22,23]. Some studies indicate an increased frequency of LOM ICR2 associated with ART, but ART was not present in Case 2. Case 2 also manifested hypomethylation at other imprinted loci (HIL): specifically, *GRB10*, *MEST* and *H19*. HIL has been described in ~25% of patients with hypomethylation of *KCNQ1OT1*[24,12], but has not been reported to confer clinical anomalies additional to those seen in BWS. Also, hypomethylation of *H19* alongside *KCNQ1OT1* is an uncommon observation in BWS (though described in SRS)[12,16], and needs further investigation for the potential influence of epigenotype on phenotype. Mutation of *NLRP2* has been reported in a similar patient[25], but it remains unclear whether trans-acting mutations are common among such patients.

It is essential to distinguish a group of patients with partial hypermethylation of the *H19* and *IGF2 DMR0* loci and normal methylation at *KCNQ1OT1*. Gain of maternal methylation at ICR1 is associated with biallelic *IGF2* expression and has been reported in approximately 5% of patients with sporadic BWS. These patients are at highest risk to develop Wilms tumor or hepatoblastoma[13]. Clinical abnormalities relevant to this molecular etiology include mainly hemihypertrophy. No previous BWS cases in the family are usually observed[26].

It is also worth mentioning that approximately 25% of patients with BWS caused by hypomethylation of *KCNQ1OT1* have additional hypomethylation in imprinted genes[12,24]. In Case 2, apparent methylation, change was observed at the following loci: *IGF2R*, *GRB10*, *MEST*, and *H19*. This hypomethylation of imprinted loci (BWS/HIL) variant seems to not confer additional clinical anomalies additional to those seen in BWS.

Fig. 1. Hemihyperplasia of the tongue.

A feature of great clinical importance in children with BWS is increased risk of tumor development, particularly Wilms tumor (43%), hepatoblastoma (20%) and adrenocortical carcinoma (7%)[27]. Studies indicate that approximately 7.5-10% of patients with BWS develop a malignancy, usually before the age of 8 years[9,28]. Hemihyperplasia and organomegaly are additional risk factors[27]. The increased risk of tumor seems to be the result of dysregulation of the expression of IGF2 and H19. The defect of IGF2 imprinting, which promotes growth, has been observed in multiple tumors[29]. In contrast, H19, encoding a biologically active non-translated mRNA, may function as a tumor suppressor[21]. GOM at maternal H19 and ICR1 is associated with loss of H19 expression and biallelic IGF2 expression and results in predisposition to tumor development[30]. In children with BWS, especially carriers of the gain of maternal methylation at ICR1, it is recommended to perform abdominal ultrasound every three months until the age of eight years and AFP measurement every three months until the age of four years[27,31]. Regular screening for tumors facilitates the earliest oncological intervention when needed. Additionally, parents of young children ought to be taught "daily caretaker abdominal examination"[31].

Another significant problem in children with BWS is neonatal symptomatic hypoglycemia, as presented in Case 1. Low glucose levels are reported in 30-50% of newborns with BWS[3]. It is caused by hyperinsulinism; however, the exact mechanism remains unclear (β-cells hyperplasia, increased IGF2 expression, defects in the adenosine triphosphate ATP-sensitive potassium channels[32]. While most cases of hypoglycemia are mild and resolve spontaneously, in 20% of patients with BWS, this metabolic complication is prolonged (duration greater than one week) and difficult to control[33]. The treatment of hypoglycemia depends on its severity and includes continuous feeding, glucose infusions, diazoxide, octreotide, or glucagon administration. In rare cases, partial pancreatomy is needed[34,35]. Immediate and proper treatment, as well as prevention of low glucose levels, is crucial for the child's life and psychomotor development in the future.

Different clinical manifestations and heterogeneous genetic anomalies in BWS may complicate the diagnosis. Further studies should be done to identify precisely the correlation between genotype and phenotype in order to provide the optimal and adequate surveillance of patients with different dysregulations of imprinting, including BWS. It is extremely important in this group of patients with increased risk of tumor development. Knowledge about the molecular etiology in all patients with BWS may significantly improve the effectiveness of the long-term follow-up, especially in the aspects of effective oncology prophylaxis.

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