Epigenotype and phenotype correlations in patients with Beckwith-Wiedemann syndrome

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Beckwith-Wiedemann Syndrome (BWS) is one of the most common overgrowth syndromes. Cancer predisposition is an important feature of this clinically heterogeneous syndrome. Patients may have fetal and early childhood overgrowth, hemihyperplasia, macroglossia, facial dysmorphic features, abdominal wall defects, visceromegaly, and anomalies of the heart and the kidneys. Various previous investigations showed that heterogeneous molecular etiology may contribute to clinical variability and that epigenotype-phenotype correlations exist in BWS. This study was performed to detect the molecular etiology in 28 patients with BWS, to search for epigenotype-phenotype correlations and to provide appropriate individualized multidisciplinary approach. Four different molecular etiology groups were determined based on testing for copy number analysis and methylation status at 11p15. Sequencing for CDKN1C mutations were also performed. Groups were compared for various clinical findings. Differences between groups were not statistically significant owing to the small number of patients in individual groups. Statistical studies for epigenotype-phenotype correlations showed significance for only anterior ear lobe creases, visceromegaly and embryonal tumors. Additionally, one interesting patient had a mesenchymal tumor. Anticipating follow-up is clinically important in BWS.

Key words: Beckwith-Wiedemann syndrome (BWS), epigenotype and phenotype correlation, imprinting, uniparental disomy, Wilms tumor.

Beckwith-Wiedemann syndrome (BWS) (OMIM #130650) is one of the best known and most common overgrowth syndromes. Incidence of BWS is about one in 13,700 live births.[1, 2] The clinical presentation is very heterogeneous and patients with mild phenotypical features may remain undiagnosed, suggesting a higher actual incidence for the disease.[3-5]

The disease is sporadic in most patients (85%) and familial in a few (15%).[4-6] Underlying molecular pathology in BWS is heterogeneous, and may be responsible from the variability of clinical features.[7,8] BWS is clinically characterized by macrosomia, macroglossia, abdominal wall defects, visceromegaly, hemihyperplasia, embryonal tumors, neonatal hypoglycemia, ear anomalies, adrenocortical cytomegaly, and renal anomalies.[9] In addition to systemic problems related to overgrowth, various anomalies involving face, abdominal wall, heart and kidneys, most important clinical problem is predisposition to malignancies.[10] Predisposition to Wilms tumor as well as to other embryonal tumors like hepatoblastoma, rhabdomyosarcoma, neuroblastoma, and adrenocortical carcinoma can be observed.[5,11,12] Tumor development may be seen in approximately 7.5% of the patients.[11,13-15] Therefore appropriately

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This manuscript is part of a thesis.

diagnosing the syndrome, through careful evaluation of all potential clinical features and molecular testing, has great clinical importance.

Molecular pathology in BWS may be caused by a variety of genetic and epigenetic alterations, affecting expression of imprinted growth regulating genes localized on chromosome 11p15.5.[4] These changes are collectively responsible from 80% of cases.[16] Two imprinted gene clusters found on chromosome 11p15.5, *IGF2/H19* domain and *CDKN1C/KCNQ1OT1/KCNQ1* domain, are functionally regulated by two imprinting centers, IC1 and IC2, respectively. Loss of normal function of these imprinted domains may lead to BWS. The mechanisms include loss of maternal methylation at IC2 in 50-60% of patients, paternal uniparental disomy (UPD) (causing biallelic paternal expression profile in both domains) in 20%, maternal *CDKN1C* mutations in 5-10%, and maternal hypermethylation of IC1 in 2-7% of patients. In less than 1% of patients, paternal 11p15 duplication causes dominance of a paternal expression profile at this region. Maternal cytogenetic anomalies like translocations or inversions may cause loss of maternal expression pattern in less than 1%. In the remaining 13-15%, underlying molecular etiology cannot be determined.

It is presumed that the clinical spectrum of variable features in BWS may be related to the heterogeneity of the underlying molecular mechanisms, as presented in Table I.[9] This current study has been conducted in search for 1) the underlying molecular etiology in a group of BWS patients using methylation sensitive multiplex ligation-dependent probe amplification (MS-MLPA) and *CDKN1C* sequencing, and 2) potential epigenotype-phenotype correlations.

Material and Methods

This study was performed between July 2011 and August 2012 at Hacettepe University, İhsan Doğramacı Children's Hospital, Division of Pediatric Genetics. Twenty-eight patients clinically consistent with BWS were included in the study. There are no absolute requisites for the clinical diagnosis of BWS. It is generally accepted that the presence of at least three major findings, or two major findings and one minor finding support a clinical diagnosis (Table II).[15] Thus, the clinical diagnoses were based on the presence of these clinical criteria.

MS-MLPA reaction, using the commercially available kit SALSA MS-MLPA probemix ME030-C1 BWS/RSS was performed as recommended by the manufacturer (MRC-Holland®, Amsterdam, The Netherlands). Cytogenetic analyses were performed on GTG-banded metaphase spreads prepared from phytohemagglutinin (PHA)-stimulated peripheral blood lymphocytes after standard culture and chromosome preparation techniques. Chromosome analyses were performed at a resolution of 550 bands. Molecular analysis for CDKN1C was performed via sequencing. For this, genomic DNA was extracted from peripheral blood lymphocytes using The QIAamp DNA Blood Mini Kit (Qiagen) according to manufacturer's recommendations. Sanger sequencing of CDKN1C gene was performed using BigDye terminator chemistry 3.1 on the 3130 Genetic Analyzer (Applied Biosystems). Primer sequences and PCR conditions are available on request.

According to the underlying molecular etiology, patients were grouped into four as follows; Group 1: loss of methylation at IC2; Group 2: gain of methylation at IC1; Group 3: paternal UPD, and Group 4: other molecular mechanisms. Patients were then examined in terms of clinical characteristics, including presence or absence of polyhydramnios, large placenta, prematurity, birth via assisted reproductive techniques (ART), high birth weight, neonatal hypoglycemia, macrosomia, anterior abdominal wall defects, macroglossia, typical facial characteristics including flat face, hypertelorism, micrognathia, facial nevus flammeus/hemangioma, anterior ear lobe crease, posterior helical pits, hemihyperplasia, nephromegaly, visceromegaly, embryonal tumors, as well as for presence of hypocalcemia, hypoglycemia, hypercholesterolemia, hypothyroidism, hypercalciuria and nephrocalcinosis.

Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) version 18.0. Descriptive statistics included count and percentage in qualitative variables; and mean, standard deviation, median, minimum and maximum values for numeric variables. Relationships between qualitative variables were studied using $\chi 2$ and Fisher's exact $\chi 2$ tests. Mann-Whitney U and

Table I. Epigenotype–Phenotype Correlations in Beckwith-Wiedemann Syndrome.[9]

Clinical features	Molecular etiology
Hemihyperplasia	UPD (most frequent) Gain of methylation at IC1 (less frequent) Loss of methylation at IC2 (less frequent)
Positive family history	CDKN1C mutation Microdeletion at IC1 Microduplication at IC2 (rare) 11p15 translocations/inversions 11p15 duplication
Cleft palate	CDKN1C mutation
Omphalocele	CDKN1C mutation Loss of methylation at IC2
Tumor type (tumor risk)	
Wilms tumor (>25%)	UPD Gain of methylation at IC1
Hepatoblastoma (>25%)	UPD Gain of methylation at IC1
Embryonal tumors other than Wilms tumor (5%)	Loss of methylation at IC2
Neuroblastoma only (<5%)	CDKN1C mutation
Developmental delay	11p15 duplication (cytogenetically visible)
Severe BWS phenotype	High levels of UPD
Monozygotic twinning	
Female and discordant	Loss of methylation at IC2
Male and discordant or concordant	UPD Gain of methylation at IC1 Loss of methylation at IC2
BWS following subfertility with or without ART	Loss of methylation at IC2

ART: assisted reproductive techniques, BWS: Beckwith-Wiedemann syndrome, CDKN1C: cyclin-dependent kinase inhibitor 1C, IC: imprinting center, UPD: uniparental disomy

Kruskal-Wallis tests were used in comparison of numerical values between groups. In all statistical tests, a p value of less than 0.05 has been considered as statistically significant.

This study was approved by the Hacettepe University Ethics Committee, and was financially supported by the Hacettepe University Scientific Research and Development Office. Verbal and written informed consents were obtained from parents of all patients.

Results

All of the patients met the clinical diagnostic criteria in Table II. Clinical features of the patients are summarized in Table III-IV.

Molecular etiology of BWS has been determined in 19 of 28 patients; 13 patients were included in Group 1 (13/28; 46%), two patients in Group 2 (2/28; 7%), four patients in Group 3 (4/28; 14%) and remaining nine patients were grouped into Group 4 (9/28; 32%). None of the patients had cytogenetic alterations, paternal duplications or *CDKN1C* mutations. None of the patients had positive family history.

Clinical and radiological findings of the patient groups are summarized in Tables III-V. Groups were statistically similar in terms of sex, age and body measurements (Table III). Statistical studies for epigenotype-phenotype correlations showed significance for only anterior ear lobe creases, visceromegaly and embryonal tumors (Table IV). Embryonal tumors consisted of one Wilms tumor in each group, and a second patient in Group 2 who had malignant mesenchymal tumor with epitheloid sarcomatous and rhabdoid components. Ages at diagnosis were 11, 19, 36 and 54 months for Wilms tumor in four

groups, respectively, and was 20 months for malignant mesenchymal tumor.

Findings in abdominal and renal ultrasonography are presented in Table V. The two patients in Group 2 had positive findings in ultrasonography (100%) and this difference was statistically significant (p=0.030). No statistically significant differences were observed regarding other parameters, as shown in Tables III-V (all p>0.05).

Discussion

BWS is a molecularly and clinically heterogeneous disorder. BWS patients benefit from a definite molecular diagnosis, allowing physicians to determine individual risks more accurately and follow patients properly. This current study showed some epigenotype-phenotype correlations exist in a cohort of 28 patients with BWS. The statistical yield would be much higher in a larger cohort, nevertheless, the results add to the existing data from previous studies.

Statistically significant differences in subgroups were observed for three clinical features only; anterior ear lobe creases, visceromegaly and embryonal tumors. Of these, anterior ear lobe creases and posterior helical pits were previously reported in 63% of patients.[5,17] In our cohort 9/28 (32.1%) of patients had anterior ear lobe creases. Statistically significant difference between groups (p=0.028) emerged since 8/13 patients (61.5%) in Group 1 had creases. This correlation had not been reported in the literature previously and should be confirmed in studies with larger groups.

Visceromegaly involving liver, spleen, pancreas, kidneys and adrenals can be found in patients with BWS. We have seen that visceromegaly affected 12/28 (42.9%) of patients, the difference between the groups being statistically significant (p=0.016). This difference was in between Group 1 (11/13 without visceromegaly) and Group 2 (both 2 with visceromegaly), although this comparison was statistically poor due to very low number of patients.

A statistically significant difference was also detected for embryonal tumor development (p=0.039). Absence of tumors in Group 1 in 12/13 and presence of tumors in 2/2 in group 2 has probably been the reason of this statistical difference. In patients with BWS, tumor development risk varies from 4% to 21%, with an average of 7.5%.[5,9,11,17-19] Tumors are mostly detected in the first decade but may

Table II. Major and Minor Findings Associated with Beckwith-Wiedemann syndrome.[15]

Major findings
Abdominal wall defect: omphalocele (exomphalos) or umbilical hernia
Macroglossia
Macrosomia (traditionally defined as height and weight >97th percentile)
Anterior ear lobe creases and/or posterior helical pits (bilateral or unilateral)
Visceromegaly of intra-abdominal organ(s); for example, liver, kidney(s), spleen, pancreas, and adrenal glands.
Embryonal tumor in childhood
Hemihyperplasia
Cytomegaly of adrenal fetal cortex, usually diffuse and bilateral
Renal abnormalities, including medullary dysplasia and later development of medullary sponge kidney
Positive family history of Beckwith-Wiedemann syndrome
Cleft palate

Minor findings
Pregnancy-related findings of polyhydramnios, enlarged placenta and/or thickened umbilical cord, premature onset of labor and delivery
Neonatal hypoglycemia
Nevus flammeus
Cardiomegaly/structural cardiac anomalies/cardiomyopathy
Characteristic facies
Diastasis recti
Advanced bone age

Table III. Gender, Age, Body Measurements of Patients.

	Group 1 (n=13)	Group 2 (n=2)	Group 3 (n=4)	Group 4 (n=9)	Total (n=28)	p
Female/male, n/n	4/9	1/1	1/3	4/5	10/18	0.934
Age, month*	47.5±34.6	48.0±24.6	82.1±23.6	45.2±34.9	51.7±33.7	0.209
Body weight, kg*	20.4±12.8	22.7±16.6	25.6±11.0	17.6±10.2	20.4±11.6	0.480
Height, cm*	101.1±24.2	100.5±26.1	113.8±10.5	95.2±22.5	101.0±22.0	0.579
Head circumference, cm*	50.0±3.1	50.0±4.2	51.8±1.5	48.9±3.9	49.9±3.2	0.494

*Data is presented as mean±standard deviation

also develop in older ages.[11, 20] The mean age of 28 patients in our study was 51.7±33.7 months, youngest patient being 9-months old and the oldest one being 120-months old. This result supports that the most important period is the first 8-10 years of life for development of embryonal malignant diseases. The rate of tumor development in our study (17.9%) was consistent with previous literature, however, as 24/28 patients were younger than 8 years of age, one can assume that overall rate could be higher after a certain period of follow-up.

Wilms tumor was found in one patient per group and malignant mesenchymal tumor, with some characteristics of both epithelioid sarcoma and rhabdoid elements, was found in the second patient in Group 2. This interesting patient in Group 2 had malignant mesenchymal tumor that developed at 20 months of age. She was diagnosed with BWS at birth on detection of macroglossia, visceromegaly, polyhydramnios, prematurity, birth weight over 97th centile, neonatal hypoglycemia and facial characteristics including micrognathia, hypertelorism and flat face. Malignant mesenchymal tumor, which

Table IV. The Clinical Findings of Patients.

Findings	Group 1 (n=13)	Group 2 (n=2)	Group 3 (n=4)	Group 4 (n=9)	Total (n=9) n (%)	p
Macrosomia	4 (30.8)	1 (50.0)	1 (25.0)	3 (33.3)	9 (32.1)	1.000
ART	2 (15.4)	-	-	-	2 (7.1)	0.690
Anterior abdominal wall defect	8 (61.5)	-	1 (25.0)	3 (33.3)	12 (42.8)	0.862
Macroglossia	12 (92.3)	1 (50.0)	3 (75.0)	5 (55.5)	21 (75.0)	0.176
Hemihyperplasia	8 (61.5)	1 (50.0)	4 (100.0)	8 (88.8)	21 (75.0)	0.225
Hemihyperplasia right side	5 (38.5)	-	3 (75.0)	7 (77.7)	15 (53.5)	0.232
Hemihyperplasia left side	3 (23.1)	1 (50.0)	1 (25.0)	1 (11.1)	6 (21.4)	0.232
Anterior ear lobe crease	8 (61.5)	-	-	1 (11.1)	9 (32.1)	0.028
Posterior helical pit	8 (61.5)	1 (50.0)	1 (25.0)	1 (11.1)	11 (39.2)	0.078
Visceromegaly	2 (15.4)	2 (100.0)	2 (50.0)	6 (66.6)	12 (42.8)	0.016
Embryonal tumor	1 (7.7)	2 (100.0)	1 (25.0)	1 (11.1)	5 (17.8)	0.039
Polyhydramnios	5 (38.5)	1 (50.0)	1 (25.0)	1 (11.1)	8 (28.5)	0.479
Large placenta	1 (7.7)	-	-	-	1 (3.5)	1.000
Prematurity	5 (38.5)	1 (50.0)	2 (50.0)	2 (22.2)	10 (35.7)	0.681
High birth weight	6 (46.2)	2 (100.0)	2 (50.0)	5 (55.5)	15 (53.5)	0.740
Neonatal hypoglycemia	6 (46.2)	1 (50.0)	1 (25.0)	2 (22.2)	10 (35.7)	0.598
Facial nevus flammeus / hemangioma	9 (69.2)	-	1 (25.0)	3 (33.3)	13 (46.4)	0.161
Facial characteristics	11 (84.6)	2 (100.0)	2 (50.0)	8 (88.8)	23 (82.1)	0.333

ART: assisted reproductive techniques
Data is presented as n (%)

takes origin from mesenchymal tissue and covers 4-8% of all childhood cancers, has not been reported previously in BWS [14]. Wilms tumor mostly accompanies UPD and gain of methylation at IC1[6,13], but still, our finding should be interpreted with caution because of small groups.

Imprinting disorders like BWS are seen more frequently in children born by ART, and in those with BWS, loss of methylation at IC2 in maternal allele is responsible.[21-24] It is still not clear whether this situation is due to the process itself or to infertility or to a combination of genetic and environmental predispositions.[24] Consistently in our study, 2/28 (7%) had a history of ART and both had maternal loss of methylation at IC2 (2/13; 15.4%).

Anterior abdominal wall defects including omphalocele, umbilical hernia, diastasis recti are among the major features of BWS, seen in 77-91% of patients.[1,5,19] In patients with omphalocele, *CDKN1C* mutations were more likely the cause of BWS, whereas loss of maternal methylation at IC2 were less likely.[9] In our cohort, omphalocele was found in groups 1 and 4, but not in other groups, in accordance with previous studies.[25] For umbilical hernia, an epigenotype-phenotype correlation has not been established previously. In Group 2, neither of the two patients had umbilical hernia or omphalocele, while a statistically significant conclusion is difficult to draw from this.

Hemihyperplasia is reported in 25% of patients with BWS.[10] In previous studies, hemihyperplasia was most commonly related to UPD, and less frequently related to IC1 and IC2 defects.[25,26] In our cohort, hemihyperplasia was present in 75% of patients (21/28). Renal anomalies involving medulla and collecting ducts are seen in 15-25% of BWS patients.[27] Various abdominorenal ultrasonographic anomalies were detected in 50% (14/28) of our patients (Table V). Differences between groups were statistically significant (p=0.030), which may be partly due to 3/13 (48%) affected in Group 1 versus 2/2 (100%) in Group 2.

Another feature of BWS is that the tissues may be affected in a mosaic form. Patients with clinically mild phenotypic features (e.g., macroglossia or umbilical hernia) may develop tumors associated with BWS in molecularly affected tissues only such as kidney and liver. [28] Considering this and the low number of patients in some of our groups, we conclude that screening for embryonal tumors must be prudently done in patients with one or more clinical features suggesting BWS. Abdominal ultrasound every 3 months up to 8 years of age and measurement of AFP level every 3 months up to 4 years of age is recommended for cancer screening in BWS patients.[11,29]

Table V. Abnormalities on Abdominorenal Ultrasounds of Patients.

Findings	Group 1 (n=13)	Group 2 (n=2)	Group 3 (n=4)	Group 4 (n=9)	Total (n=28)
Hepatomegaly	2 (15.4)	1 (50.0)	2 (50.0)	4 (44.4)	9 (32.1)
Nephromegaly	1 (7.7)	2 (100.0)	2 (50.0)	3 (33.3)	8 (28.5)
Adrenocortical thickening	-	-	2 (50.0)	-	2 (7.1)
Splenomegaly	-	-	1 (25.0)	1 (11.1)	2 (7.1)
Collecting system dilatation	-	-	1 (25.0)	-	1 (3.5)
Hydronephrosis	-	-	-	1 (11.1)	1 (3.5)
Nephrocalcinosis	-	-	1 (25.0)	1 (11.1)	2 (7.1)
Renal medullary dysplasia	-	-	1 (25.0)	-	1 (3.5)
Wilms tumor	1 (7.7)	1 (50.0)	1 (25.0)	1 (11.1)	4 (14.2)
Liver cyst	-	-	-	1 (11.1)	1 (3.5)
Accessory spleen	-	-	-	1 (11.1)	1 (3.5)
Normal	10 (76.9)*	-	1 (25.0)	1 (11.1)	12 (42.8)

Data is presented as n (%)
*: Ultrasound abnormalities in Group 1 were significantly less common, compared to other Groups (p=0.030)

Molecular studies should also be performed for patients with isolated hemihyperplasia. In the literature, 5.9% increase in the risk of tumor development has been reported in patients with isolated hemihyperplasia.[30] Choyke et al.[31] has reported that when patients with BWS or isolated hemihyperplasia are not screened by abdominal US for tumor every 4 months or more frequently, at the time of diagnosis high grade (grade 3 or 4) Wilms tumor rates are greater than screened population. Minimal clinical features of BWS, such as isolated hemihyperplasia, should be carefully managed and molecular genetic tests should be recommended.[28]

This study aimed to investigate the relationship between phenotypic expression of BWS and specific molecular subgroups. Although relative percentages of the molecular subgroups were consistent with the previously reported studies, small number of the study population was a limitation of this study. Studies covering larger groups of patients may render the assessment of priority of major and minor clinical features in BWS and clinical diagnosis of the syndrome may be revised.

In conclusion, investigating the underlying molecular etiology in BWS should be recommended even in patients with few clinical characteristics, since molecular changes may be detected even in patients with isolated hemihyperplasia. Embryonal malignancies, particularly Wilms tumor should be screened in all patients with BWS, as these are seen more commonly than in general population. Rare embryonal tumors like malignant mesenchymal tumor may as well be encountered in patients with BWS. A multidisciplinary approach is essential in follow-up of patients with BWS, and intra-abdominal pathology other than tumors should also be investigated.

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