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Differential microscopic finding and glucose transporter 3 expression in terminal chorionic villi among birth weight-discordant twin placentas

Mina Lee[1], Song-Yi Choi[2], Byung-Hun Kang[3], Heon-Jong Yoo[3], Soo-Youn Song[3], In-Ock Seong[2], Kwang-Sun Suh[2] and Kyung-Hee Kim[2,4]

[1]Department of Obstetrics and Gynecology, [2]Department of Pathology, [4]Cancer Research Institute, Chungnam National University, School of Medicine and [3]Department of Obstetrics and Gynecology, Chungnam National University Hospital, Daejeon, Republic of Korea

Summary. Objective: To evaluate differences in microscopic findings and glucose transporter 3 (GLUT3) expression in terminal chorionic villi (TV) among birth weight-discordant twin (BWDT) placentas compared with the birth weight-concordant twin (BWCT) placentas.

Methods: We retrospectively studied a cohort of 26 BWDT, 10 BWCT, 10 pre-eclampsia singleton and 10 normal singleton pregnancies. Placentas were scored for the percentage of TV, the percentage of TV with syncytial knots, the presence of capillary branching patterns of TV, the capillary to terminal villous ratios, the membranous expression of GLUT3 and the nuclear expression of HIF-1α in trophoblasts and capillary endothelial cells of TV using immunohistochemistry. The clinical characteristics and microscopic findings were analyzed and compared.

Results: BWDT placentas exhibited differential percentages of TV, percentages of TV with syncytial knots, capillary to terminal villous ratios, expression of HIF-1α in capillary endothelial cells and expression of GLUT3 in trophoblasts and capillary endothelial cells of TV among each twin pair compared with BWCT placentas (P=0.003, P=0.022, P=0.037, P=0.007, P=0.046 and P=0.002, respectively). Pre-eclampsia singleton placentas exhibited higher GLUT3 expression in trophoblasts, higher HIF-1α expression in capillary endothelial cells of TV and high capillary to terminal villous ratios compared with normal singleton placentas (P=0.001, P<0.001 and P=0.001, respectively).

Conclusions: We observed a strong relationship between characteristics of adaptive change to hypoxia (GLUT3 expression, TV and syncytial knotting and higher capillary to terminal villous ratios) and BWDT pregnancy but not BWCT pregnancy.

Key words: Birth weight, Twin, Placenta, Trophoblast, Glucose transporter 3

Offprint requests to: Kyung-Hee Kim or Kwang-Sun Suh, Department of Pathology, Cancer Research Institute, Chungnam National University, School of Medicine, Jung-gu, Daejeon, 301-747 South Korea. e-mail: phone330@cnu.ac.kr or kssuh@cnu.ac.kr

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Introduction

Twin pregnancies are correlated with significantly higher rates of perinatal morbidity and mortality than singleton pregnancies, and discordant in utero growth presents a considerable risk (Sonntag et al., 1996). Perinatal morbidities associated with birth weight discordancy include intrauterine growth restriction, preterm birth, infection, and stillbirth (Fraser et al., 1994).

Trans-placental transport of nutrients is a well-known determinant of fetal growth, and altered glucose transport causes subnormal growth in both singleton and twin pregnancies. Glucose from the maternal circulation is the essential source of fetal and placental glucose due to the absence of fetal gluconeogenesis (Marconi et al., 1993). The passage of glucose across cell membranes is facilitated by the membrane-spanning glucose transporter (GLUT) family, which contains 14 known isoforms. GLUT1 is the most common isoform expressed in a wide variety of human tissues, and GLUT3 has been reported in tissues with high requirements for glucose metabolism, including neurons, sperm, preimplantation embryos, and an array of

carcinoma cell lines (Simpson et al., 2008). Janzen et al. demonstrated that increased GLUT3 expression in maternal trophoblasts in growth-restricted singleton placentas was correlated with hypoxia inducible transcription factor-1α (HIF-1 α) and suggested that hypoxia could facilitate GLUT3 expression in trophoblasts (Janzen et al., 2013).

The histological features correlating with GLUT3 expression in birth weight-discordant twins (BWDTs) are not known. We hypothesized that differential expression of GLUT3 between BWDT placentas might predict the presence of fetal hypoxia in BWDTs. To examine this hypothesis, we evaluated a cohort of 26 BWDT cases, 10 birth weight-concordant twin (BWCT) cases, 10 pre-eclampsia singleton cases and 10 normal singleton cases for the association of histological features and GLUT3 expression on terminal chorionic villi (TV) using GLUT3 and HIF-1α immunostainings in trophoblasts and maternal TV capillary endothelial cells.

Materials and methods

Patients and tissue collection

A consecutive series of BWDTs (n=26), BWCTs (n=10), pre-eclampsia singletons (n=10) and normal singletons (n=10) were collected at the Department of Obstetrics and Gynecology and Pathology at Chungnam National University Hospital between 2005 and 2013. Placental tissues were collected from 26 pairs of BWDT (8 diamnionic-monochorionic and 18 diamnionic-dichorionic placentas), 10 pairs of BWCT (3 diamnionic-monochorionic and 7 diamnionic-dichorionic placentas), 10 cases of preeclampsia singleton pregnancies and 10 cases of normal singleton pregnancies. All twin placentas were not complicated by twin-twin transfusion syndrome, abnormal umbilical cord insertion, or placental shape aberrations. Inter-twin birth weight discordance was defined as a difference in birth weight >20%, calculated using a percentage of the weight of the larger twin; (larger twin weight-smaller twin weight)/larger twin weight x100 (Demissie et al., 2002; Amaru et al., 2004). Our 26 cases of BWDTs ranged from 22% to 46% weight disconcordance. The control BWCTs were not associated with birth weight discordance (ranged from 3.8% to 14.8%), twin-twin transfusion, or placental shape aberrations. Clinical information for the groups is presented in Table 1. All specimens were collected from paraffin-embedded delivery placentas. The paraffin embedded samples were taken from mid-placenta to central placenta, were free of visible infarction or calcification, and included the chorionic plate to basal plate on a vertical section. This study was approved by the Institutional Review Board of Chungnam National University Hospital.

Immunohistochemistry

For immunohistochemical study, multiple sections were reviewed from the placenta of each baby, and one

Table 1. Analysis of clinicopathological variables, placental pathology and the expression of GLUT3 and HIF-1α among birth weight-discordant and birth weight-concordant twins.

Characteristics	Birth weight		
	concordance	discordance	P
Maternal weight (Kg)			0.706[a]
No.	10	26	
Mean	55.50±7.835	56.68±7.739	
Gestational age (weeks)			0.001[a]
No.	10	26	
Mean	36.84±0.648	35.22±1.651	
Maternal hemoglobin			0.639[a]
No.	10	26	
Mean	12.00±1.039	12.06±2.046	
Diff. S/D ratio of UA.			0.157[a]
No.	8	12	
Mean	0.47±0.227	0.79±0.576	
>3 S/D ratio of smaller twin.	8(100.0)	15(100.0)	0.050[b]
– (%)	8(100.0)	9(60.0)	
+ (%)	0(0.0)	6(40.0)	
Dichorionic	10(100.0)	26(100.0)	1.000[b]
– (%)	3(30.0)	8(30.8)	
+ (%)	7(70.0)	18(69.2)	
Hypertension	10(100.0)	26(100.0)	0.023[c]
– (%)	10(100.0)	19(73.1)	
+ (%)	0(0.0)	7(26.9)	
Diabetes mellitus	10(100.0)	26(100.0)	0.416[c]
– (%)	10(100.0)	25(96.2)	
+ (%)	0(0.0)	1(3.8)	
Diff. GLUT3 on TB.	10(100.0)	26(100.0)	0.046[b]
– (%)	9(90.0)	14(53.8)	
+ (%)	1(10.0)	12(46.2)	
Diff. GLUT3 on cap.	10(100.0)	26(100.0)	0.002[b]
– (%)	10(100.0)	11(42.3)	
+ (%)	0(0.0)	15(57.7)	
Diff. HIF-1α on TB.	10(100.0)	26(100.0)	0.709[b]
– (%)	5(50.0)	16(61.5)	
+ (%)	5(50.0)	10(38.5)	
Diff. HIF-1α on cap.	10(100.0)	26(100.0)	0.007[b]
– (%)	8(80.0)	7(26.9)	
+ (%)	2(20.0)	19(73.1)	
Diff. terminal CV.	10(100.0)	26(100.0)	0.003[b]
– (%)	10(100.0)	12(46.2)	
+ (%)	0(0.0)	14(53.8)	
Diff. syncytial knot	10(100.0)	26(100.0)	0.022[b]
– (%)	9(90.0)	11(42.3)	
+ (%)	1(10.0)	15(57.7)	
Diff. cap. of terminal CV.	10(100.0)	26(100.0)	0.037[c]
– (%)	10(100.0)	20(76.9)	
+ (%)	0(0.0)	6(23.1)	
Diff. br. of terminal CV.	10(100.0)	26(100.0)	0.645[b]
– (%)	9(90.0)	20(76.9)	
+ (%)	1(10.0)	6(23.1)	

[a], Mann Whitney U-test; [b], Fisher's exact test; [c], Likelihood ratio; Diff. S/D ratio of UA, differential value of antenatal systolic/diastolic ratio of the umbilical cord artery upon delivery between each twin pair; S/D ratio of smaller twin, systolic/diastolic ratio of umbilical cord artery of the smaller baby; Diff. GLUT3 in TB, differential grade of GLUT3 expression on the trophoblasts of terminal chorionic villi between each twin pair; Diff. GLUT3 on cap., differential grade of GLUT3 expression on the capillaries of terminal chorionic villi between each twin pair; Diff . HIF-1α in TB, differential grade of HIF-1α expression on the trophoblasts of terminal chorionic villi between each twin pair; Diff. HIF-1α on cap ., differential grade of HIF-1α expression on the capillaries of terminal chorionic villi between each twin pair; Diff. syncytial knot, differential number of terminal chorionic villi with syncytial knots between each twin pair; Diff. terminal CV, differential number of terminal chorionic villi between each twin pair; Diff. cap. of terminal CV, differential capillary to terminal chorionic villous ratio between each twin pair; and Diff. br. of terminal CV, differential capillary branching pattern of terminal chorionic villi between each twin pair.

representative paraffin block including both the chorionic plate and basal plate on a vertical section was selected. A heat-mediated antigen retrieval was performed with 10 mmol/L sodium citrate (pH 6.0) (Dako, Glostrup, Denmark) for 15 min using a pressure cooker at full power for 3 minutes. Sections were incubated at ambient temperature for 60 min with rabbit polyclonal anti-glucose transporter GLUT3 antibody (9 μg/ml, 1:200; catalog #AB15311, Abcam Inc., Cambridge, MA, USA) and rabbit polyclonal anti-HIF-1α antibody (5 μg/ml, 1:200; catalog #NB100-479, Novus Biologicals, Littleton, CO, USA). Sections were then incubated in Dako EnVision+ System-HRP (DAB) For Use with Rabbit Primary Antibodies (Dako, Glostrup, Denmark) for 20 min at room temperature. The chromogen was then developed for 2 minutes, and slides were counterstained with Meyer hematoxylin. In negative controls, polyclonal rabbit IgG antibody (catalog #AB27472, prediluted; Abcam, Cambridge, Massachusetts) was used as a substitute for the primary antibodies as an isotype-matched control antibody.

Membranous expression of GLUT3 and nuclear expression of HIF-1α were observed in trophoblasts and capillary endothelial cells of TV on the maternal side because the TV is the primary site of fetomaternal exchange for the transfer of oxygen (Lackman et al., 2001). Because the stained cells exhibited invariable intensity, the staining intensity was not graded. We used the modified Allred et al. method for evaluating the proportion of stained trophoblasts and TV capillary endothelial cells in each stained slide (Allred et al., 1998). The proportional scores were: score 0, no staining; score 1, >0 to 1/100 stained; score 2, >1/100 to 1/10 stained; score 3, >1/10 to 1/3 stained; score 4, >1/3 to 2/3 stained; and score 5, >2/3 to 1 stained. Ten low-power fields (×100 magnification) were examined and scored by median value (Altshuler, 1984; Ogino and Redline, 2000). A median value difference in GLUT3 or HIF-1α expression greater than 0 between each twin pair was defined as a differential grade using the following formula: |GLUT3 expression score of one twin baby – GLUT3 expression score of the other twin baby|. Each slide was examined separately and scored by two pathologists (K. H. K. and K. S. S.). Discrepancies in scores were discussed to obtain a consensus.

Placental histological examination

Two representative sections of mid-placenta to central placental parenchyma including both the chorionic plate and basal plate on a vertical section were analyzed. Routine hematoxylin & eosin (H&E)-stained sections were scored to evaluate the percentage of TV of the cross-section, percentage of TV possessing syncytial knots, the capillary branching pattern of the TV and the capillary to terminal villous ratio. Histological examination was performed by two pathologists (K. H. K. and K. S. S.) while blinded to clinical characteristics, including birth weight discordant history, gestational age, and clinical outcomes.

The TV percentages of the cross-sections were scored as follows: score 1, <40%; score 2, 40% to <60%; and score 3, ≥60%. The percentage of TV possessing syncytial knots were scored as followed: score 1, <30%; score 2, 30% to <50%; and score 3, ≥50% (Tenney and Parker, 1940; Loukeris et al., 2010). The TV capillary branching pattern was scored as follows: score 1, predominant branch; score 2, grapelike; and score 3, non-branching (Kingdom and Kaufmann, 1997). The capillary to terminal villous ratio was scored as the median value of raw data.

Counts were performed on 10 low-power fields (×100 magnification) in forward half from the basal plate and expressed as median values. Score differences greater than 0 in the TV percentage, percent of TV possessing syncytial knots and the TV capillary branching patterns between each twin pair was defined as a differential grade. More than 1 difference score in the capillary to terminal villous ratio between each twin pair was defined as a differential grade.

Statistics

Associations between differential GLUT3 or HIF-1α expression on trophoblasts and TV capillary endothelial cells and birth weight discordance were assessed using Fisher's exact test. The clinicopathological variables were analyzed for statistical significance using the Mann-Whitney U-tests, Likelihood ratio and Fisher's exact test. Statistical significance was defined as $P<0.05$ (IBM SPSS 21 statistics; SPSS Inc., Chicago, IL, USA).

Results

Clinical features

The average gestational age of the BWDT group and BWCT group was 35.22 weeks (30.1-38.1 weeks) and 36.84 weeks (36.0-38.0 weeks), respectively (P=0.001). Seven cases among 26 cases of BWDTs were associated with maternal hypertension, whereas no case among 10 cases of BWCT was associated with maternal hypertension (P=0.023). Fifteen smaller babies and 12 larger babies of 26 BWDTs and 8 pairs of 10 BWCTs at 29-37 weeks of gestational age underwent antenatal umbilical cord artery Doppler study. The systolic/diastolic ratio of the umbilical cord artery is a measure of resistance in the placental vasculature (Moon, 1999). The mean differential systolic/diastolic ratio of the umbilical cord artery between each twin pair of BWDTs was higher than that of BWCTs, but the differences did not reach statistical significance (P=0.157). Six smaller babies of 15 BWDTs exhibited umbilical cord artery systolic/diastolic ratios greater than 3.0, whereas 0 out of 8 pairs of BWCT receiving antenatal umbilical cord artery Doppler study exhibited rates greater than 3.0 (P=0.050) (Table 1). Five among the 7 hypertensive women pregnant with BWDT had pre-eclampsia. Two among the 26 women pregnant with BWDT exhibited uterine leiomyoma, and two among 26 women pregnant

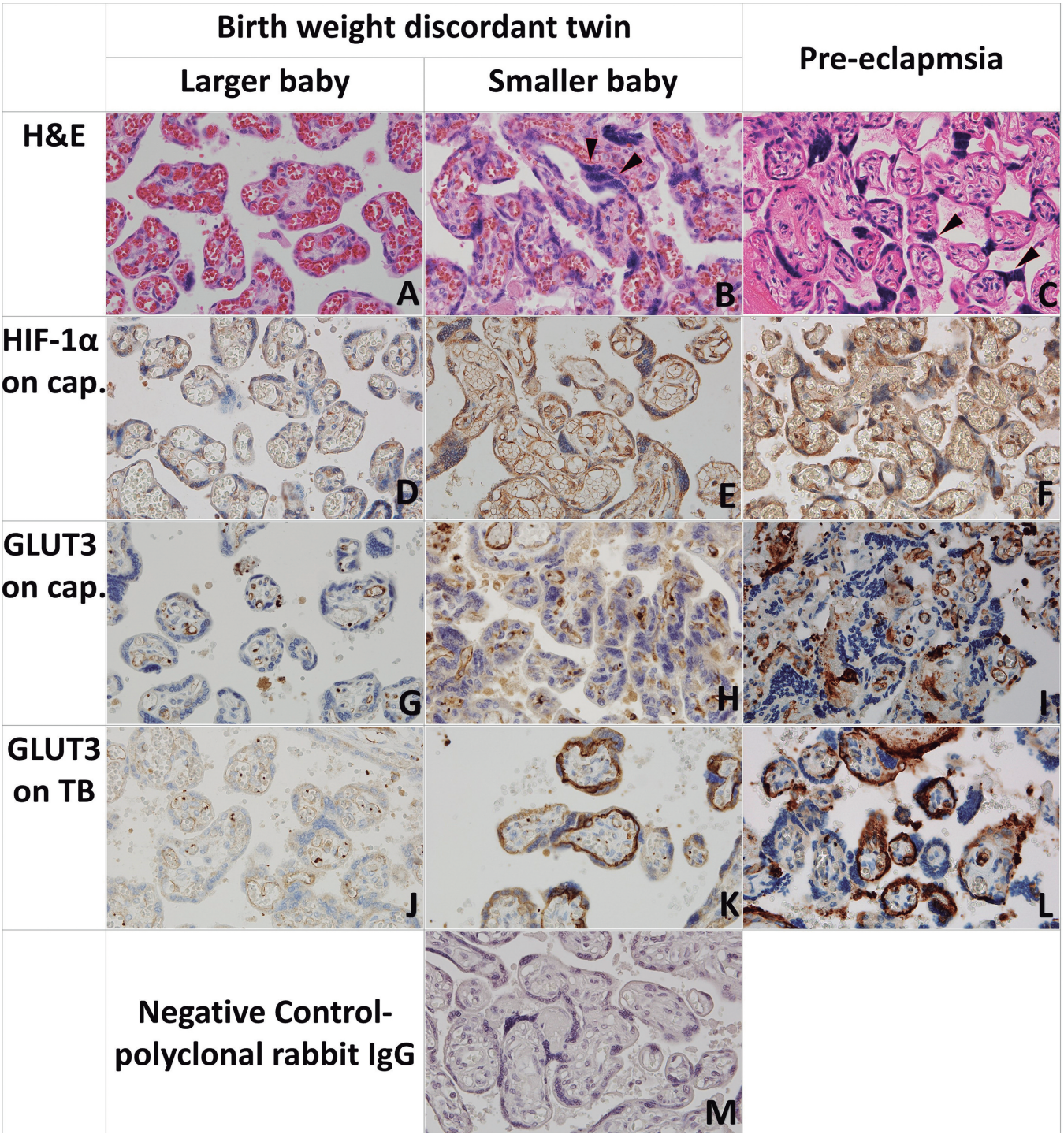

Fig. 1. Representative micrographs of terminal chorionic villi. **A, B.** Differential number of terminal villi (TV) with syncytial knots between each pair of birth weight discordant twin (BWDT) placentas. A higher rate of syncytial knots (arrowheads) in the smaller baby placenta was observed compared with the larger baby placenta. **C.** Increased number of syncytial knot (arrowheads) formation in the pre-eclampsia placenta. **D, E.** Differential HIF-1α nuclear expression on capillary (cap.) endothelial cells of terminal villi between each pair of BWDT placentas. Higher expression of HIF-1α in the smaller baby placenta compared with the larger baby placenta. **F.** Increased expression of HIF-1α on capillary endothelial cells of terminal villi of pre-eclampsia placenta. **G, H.** Differential GLUT3 expression on capillary endothelial cells of terminal villi between each pair of BWDT placentas. Higher expression of GLUT3 in the smaller baby placenta compared with the larger baby placenta. **I.** Increased expression of GLUT3 on capillary endothelial cells of terminal villi of pre-eclampsia placenta. **J, K.** Differential GLUT3 expression on trophoblasts of the basal membrane of syncytiotrophoblasts in terminal villi between each pair of BWDT placentas. Higher expression of GLUT3 in the smaller baby placenta compared with the larger baby placenta. **L.** Increased expression of GLUT3 trophoblasts of terminal villi in pre-eclampsia placenta. **M.** Negative control immunohistochemical staining, polyclonal rabbit IgG antibody as an isotype-matched control antibody for the anti-GLUT3 and anti-HIF-1α. x 400

with BWDT exhibited adenomyosis (Table 2). None of the 26 BWDT cases, 10 BWCT cases, 10 pre-eclampsia singleton cases and 10 normal singleton cases were associated with antepartum stillbirth or neonatal death.

Correlation of birth weight discordance with terminal villous maldevelopment: Maturation of TV, proliferation of terminal villous capillaries, and TV capillary branching pattern

Fourteen among 26 pairs of BWDT placenta exhibited differential TV volume, whereas none of the ten pairs of BWCT placenta exhibited such differences (P=0.003). Six of the 26 pairs of BWDT placenta exhibited a greater than 2-fold difference in the number of terminal villous capillaries in each of 10 TV in 10 fields with a 10x objective, whereas no difference was observed among BWCT placentas (P=0.037). Fifteen cases among 26 pairs of BWDT placenta exhibited a differential TV syncytial knot grade between each twin pair, whereas only one among the 10 pairs of BWCT placenta exhibited a differential grade (P=0.022). The

Table 2. Additional clinicopathological variables and GLUT3 expression among birth weight-discordant twin pregnancies.

No	BWDT	S/D larger twin	S/D smaller twin	Maternal Age (yr)	Maternal H/T	Other diagnosis	Gest.Age (Wk)	larger twin Wt (g)	smaller twin Wt (g)	WD (%)	placental type	GLUT3 on TB of larger twin	GLUT4 on TB of smaller twin	Diff. GLUT3 on TB	GLUT3 on cap. of larger twin	GLUT3 on cap. of smaller twin	Diff. GLUT3 on cap
1	Yes	3,19	2,26	28	Yes	HBV carrier	34,1	2030	1590	21,7	dichorionic	0	0	No	2	3	Yes
2	Yes	2,65	3,85	35	Yes		36,0	2240	1720	23,2	dichorionic	0	1	Yes	1	1	No
3	Yes	2,02	2,87	32	No		35,2	2610	1710	34,5	dichorionic	0	1	Yes	1	3	Yes
4	Yes	2,12	2,55	31	No	Myoma Ut	36,0	3100	2180	29,7	dichorionic	0	3	Yes	1	2	Yes
5	Yes	2,49	3,08	32	No	Myoma Ut	36,5	2910	2200	24,4	dichorionic	0	0	No	2	2	No
6	Yes	2,48	2,15	36	No		36,5	2680	2030	24,3	dichorionic	0	2	Yes	2	2	No
7	Yes			36	No	Preterm labor	35,2	2230	1490	33,2	dichorionic	0	0	No	2	3	Yes
8	Yes			29	No		37,0	2450	1530	37,6	dichorionic	0	1	Yes	2	3	Yes
9	Yes		3,19	30	Yes	Pre-E	35,1	2610	1770	32,2	dichorionic	0	0	No	1	2	Yes
10	Yes			27	No		37,3	2800	1920	31,4	dichorionic	0	1	Yes	1	1	No
11	Yes			38	Yes	Prev C/S, Pre-E	34,6	2370	1340	43,5	dichorionic	0	0	No	1	3	Yes
12	Yes			32	No	Preterm labor	30,1	1400	780	44,3	Mono	1	1	No	0	1	Yes
13	Yes		7,39	31	No	Oligohydramnios	32,5	1730	930	46,2	dichorionic	1	1	No	3	3	No
14	Yes			39	No	Hypothyroidism	35,6	2450	1910	22,0	dichorionic	0	1	Yes	0	2	Yes
15	Yes		2,47	32	No	Preterm labor	33,1	2280	1510	33,8	dichorionic	0	1	Yes	2	2	No
16	Yes	2,46	3,8	31	No	Adenomyosis, PI accreta	36,2	2290	1660	27,5	Mono	0	1	Yes	1	1	No
17	Yes			36	No	R/O TTTS	38,1	3040	2370	22,0	dichorionic	0	0	No	1	1	No
18	Yes	2,32	2,44	28	No		37,0	2780	2150	22,7	dichorionic	0	0	No	1	2	Yes
19	Yes	2,94	2,56	30	No	Preterm labor	34,3	2130	1590	25,4	dichorionic	1	2	Yes	1	2	Yes
20	Yes	2,79	5	28	No	R/O TTTS, Oligohydramnios	35,0	1880	1280	31,9	Mono	0	0	No	2	2	No
21	Yes			38	No	Prev C/S, Polyhydramnios	35,4	2640	2010	23,9	Mono	0	0	No	1	1	No
22	Yes	2,43	1,74	32	Yes	Pre-E, R/O Polyhydramnios	35,1	2610	2160	17,2	Mono	0	0	No	2	2	No
23	Yes			28	No	Preterm labor	34,2	2390	1460	38,9	Mono	0	0	No	1	2	Yes
24	Yes			31	No	preterm labor, R/O TTTS, Adenomyosis	35,3	2620	2020	22,9	Mono	0	1	Yes	1	2	Yes
25	Yes	1,83	2,25	31	Yes	Pre-E, R/O TTTS	36,1	2510	1760	29,9	Mono	1	1	No	1	2	Yes
26	Yes			41	Yes	Pre-E, pelvic adhesion	34,2	2720	1560	42,6	dichorionic	0	1	Yes	2	3	Yes
27	No			28	No		36,5	2430	2310	4,9	Mono	1	1	No	1	1	No
28	No			30	No		37,0	2830	2540	10,2	dichorionic	1	2	Yes	1	1	No
29	No	1,58	2,35	36	No		37,0	2620	2380	9,2	dichorionic	0	0	No	1	1	No
30	No	2,32	2,42	32	No		38,0	2810	2600	7,5	dichorionic	0	0	No	2	2	No
31	No	2,33	2,71	33	No		36,0	3100	2700	13,0	dichorionic	0	0	No	1	1	No
32	No	2,24	2,56	30	No		37,5	2830	2410	12,9	dichorionic	0	0	No	1	1	No
33	No	1,62	1,94	34	No		36,3	2540	2300	9,4	Mono	1	1	No	1	1	No
34	No	2	2,63	37	No		37,4	2440	2090	14,3	Mono	0	0	No	1	1	No
35	No	1,74	2,36	33	No		36,5	2720	2420	11,0	dichorionic	0	0	No	1	1	No
36	No	1,97	2,62	34	No	maternal arhythmia	36,2	2860	2750	3,8	dichorionic	0	0	No	1	1	No

BWDT, birth weight discordant twin; S/D, systolic/diastolic ratio of antenatal umbilical cord artery upon delivery; H/T, hypertension; WD, weight discordancy; Diff. GLUT3 on TB., differential grade of GLUT3 expression on trophoblasts of terminal chorionic villi between each twin pair; Diff. GLUT3 on cap., differential GLUT3 expression on capillaries of terminal chorionic villi between each twin pair; HBV, hepatitis B virus; Ut, uterus; Pre-E, pre-eclamsia; Prev C/S, previous caesarean section; R/O, rule out; TTTS, Twin to twin transfusion syndrome

shapes of the TV villi on cross-section were classified as multiple indented branching TV, grapelike TV and poorly branched TV. The differential TV shapes between BWDTs did not reach statistical significance relative to the BWCT placenta group (P=0.645) (Table 1).

Differential GLUT3 or HIF-1α expression among birth weight-discordant twin placentas

We investigated 26 pairs of BWDT placenta and 10 pairs of control BWCT placenta. Membranous GLUT3 expression was observed in the basal membranes of the syncytiotrophoblast, between the syncytiotrophoblast and cytotrophoblast, and on the capillary endothelial cells of TV on the maternal side (Fig. 1). The differential GLUT3 expression in trophoblasts and TV capillary endothelial cells between each twin pair of BWDT placentas attained statistical significance relative to the BWCT placenta group (P=0.046 and P=0.002, respectively). Nuclear HIF-1α expression was observed in the trophoblasts and capillary endothelial cells of TV on the maternal side (Fig. 1). The differential HIF-1α expression in TV capillary endothelial cells between each twin pair of BWDT placentas was statistically significant relative to the BWCT placenta group (P=0.007), but the difference in trophoblasts of TV did not reach statistical significance (P=0.709) (Table 1). GLUT3 expression was elevated in trophoblasts and capillary endothelial cells of TV in the placentas of smaller babies in BWDT pregnancies (P=0.001 and P=0.002, respectively). The expression of HIF-1α was higher in the capillary endothelial cells of TV in the placentas of smaller babies in BWDT pregnancies (P=0.004), but not in trophoblasts (P=0.298) (Table 3).

Table 3. Analysis of GLUT3 and HIF-1α expressions among smaller baby placentas compared with larger baby placentas in 26 birth-weight discordant twins.

Characteristics	Birth weight-discordant twins		
	Smaller baby	Larger baby	P
GLUT3 on TB.			0.001[a]
No.	26	26	
Mean	0.73±0.778	0.15±0.368	
GLUT3 on cap.			0.002[a]
No.	26	26	
Mean	2.04±0.7209	1.35±0.689	
HIF-1α on TB.			0.298[a]
No.	26	26	
Mean	2.85±1.008	3.120±1.033	
HIF-1α on cap.			0.004[a]
No.	26	26	
Mean	3.58±1.027	2.69±0.884	

[a], Mann Whitney U-test; GLUT3 on TB., grade of GLUT3 expression on trophoblasts of terminal chorionic villi; GLUT3 on cap., grade of GLUT3 expression on capillaries of terminal chorionic villi; HIF-1α on TB., grade of HIF-1α expression on trophoblasts of terminal chorionic villi; HIF-1α on cap., grade of HIF-1α expression on capillaries of terminal chorionic villi

Differential terminal villous capillary proliferation, GLUT3 expression and HIF-1α expression between pre-eclampsia singleton and normal singleton placentas

Pre-eclampsia singleton placentas exhibited statistically higher expression of GLUT3 in TV trophoblasts, higher expression of HIF-1α in TV capillary endothelial cells and a higher capillary to terminal villous ratio compared with normal singleton placentas (P=0.001, P<0.001 and P=0.001, respectively). The differential expression of GLUT3 in the TV capillary endothelial cells between pre-eclampsia and normal singleton placentas did not attain statistical significance (P=0.247) (Table 4).

Discussion

This study demonstrates that placentas from BWDTs relative to BWCTs differ in the following four characteristics: 1.) percentage of TV among cross-sections; 2.) percentage of TV possessing syncytial knots; 3.) capillary to terminal villous ratio; and 4.) GLUT3 expression in trophoblasts and TV capillary endothelial cells on the maternal side. This is the first

Table 4. Analysis of GLUT3 and HIF-1α expressions among pre-eclampsia singleton placentas compared with normal singleton placentas.

Characteristics	Pre-eclampsia		
	Negative	Positive	P
GLUT3 on TB.			0.001[a]
No.	10	10	
Mean	0.40±0.516	1.70±0.675	
GLUT3 on cap.			0.247[a]
No.	10	10	
Mean	1.80±0.919	2.30±0.823	
HIF-1α on TB.			0.165[a]
No.	10	10	
Mean	2.60±0.699	3.20±0.919	
HIF-1α on cap.			<0.001[a]
No.	10	10	
Mean	3.30±0.675	4.70±0.483	
Cap. of terminal CV.			0.001[a]
No.	10	10	
Mean	3.30±0.483	5.30±1.252	
Syncytial knot			<0.001[a]
No.	10	10	
Mean	1.30±0.483	2.50±0.527	

[a], Mann Whitney U-test; GLUT3 on TB., grade of GLUT3 expression on trophoblasts of terminal chorionic villi; GLUT3 on cap., grade of GLUT3 expression on capillaries of terminal chorionic villi HIF-1α on TB., grade of HIF-1α expression on trophoblasts of terminal chorionic villi; HIF-1α on cap., grade of HIF-1α expression on capillaries of terminal chorionic villi; Cap. of terminal CV, grade of capillary to terminal chorionic villous ratio; Syncytial knot, number terminal chorionic villi possessing syncytial knots

study, to our knowledge, to evaluate the association in the differential placental microscopic characteristics and GLUT3 expression between BWDT pairs compared with BWCT pairs.

Placentas associated with intrauterine growth restriction in singleton pregnancies exhibit gross and microscopic morphological changes in chorionic villi associated with fetal hypoxia (Salafia et al., 2006). Placental histological examination is essential in the evaluation of intrauterine growth restriction because the placenta is an organ of maternal-fetal oxygen and nutrient exchange; after leaving the spiral arteries, maternal blood circulates through the intervillous space, flowing directly around the villi. Fetal oxygenation is the primary determinant of fetal growth, and fetoplacental capillaries within the TV are separated from the maternal blood by syncytiotrophoblasts and the vasculo-syncytial membrane (Lackman et al., 2001). The origins of fetal hypoxia fall into three categories: (1) preplacental hypoxia caused by maternal hypoxic states, such as maternal anemia or pregnancy at high altitude; (2) uteroplacental hypoxia caused by underperfusion of normally oxygenated maternal blood into the uteroplacental tissue, such as pre-eclampsia at term or IUGR in late pregnancy with preserved end-diastolic flow velocity in the umbilical arteries; and (3) postplacental hypoxia caused by major defects in fetoplacental perfusion, such as antepartum stillbirth, fetoplacental vascular obstruction or IUGR with absent end-diastolic flow velocity in the umbilical arteries (Kingdom and Kaufmann, 1997).

BWDTs in the absence of twin-twin transfusion syndrome are largely attributed to unequal placental sharing in monochorionic twins, and dichorionic twins may be complicated by placental pathologies localized to only one placenta (Bleker et al., 1988; Breathnach et al., 2011). BW-discordant dichorionic twins could be the result of differences in placental mass or differences in placental parenchymal lesions, such as chronic villitis or massive perivillous fibrin depositions, or differences in karyotypes or other congenital malformations (Eberle et al., 1993; Nikkels et al., 2008). However, there is a paucity of data in the literature on the microscopic chorionic villous lesions in BWDTs compared with BWCTs. We evaluated the microscopic findings of TV in both BWDT and BWCT placentas. The gestational age exhibits a positive correlation with predominantly branching angiogenesis of TV villi in the smaller BWDTs (P=0.049). Predominantly branching TV capillary pattern is associated with increased syncytial knotting, a consequence of trophoblastic flat sectioning (Tenney and Parker, 1940). In postplacental hypoxia state, the failure of TV capillaries to branch and increased intraplacental oxygen concentration prevent the fetus from receiving sufficient oxygen (Hitschold et al., 1993; Kingdom and Kaufmann, 1997; Krebs et al., 1996). In contrast to postplacental hypoxia, preplacental or uteroplacental hypoxia with a reduction in villous oxygen content results in placental adaptation in the form of predominantly branching TV angiogenesis with increased syncytial knotting. Placental angiogenesis is dependent upon the intraplacental oxygenation status (Kingdom and Kaufmann, 1997). An altered capillary to villous ratio is a widely assumed characteristic of hypoxic placentas. The proliferation of villous capillaries is accepted as an adaptation to chronic oxygen deficiency. Increased syncytial knotting is generally accepted as a mark of perfusion compromise when more than 30% of TV possess syncytial knots [12, 13]. Fifteen of the 26 BWDT pairs exhibited an increased syncytial knotting (≥30% TV possessing syncytial knot) in the placenta of the smaller baby, whereas 2 cases of the 10 BWCTs exhibited similar findings. Four placentas among the 15 placentas with increased syncytial knotting heterogeneously exhibited predominantly branching TV angiogenesis. None of the cases in this study were associated with antepartum stillbirth or neonatal death. In antenatal umbilical artery Doppler study, six smaller babies of 15 BWDTs exhibited systolic/diastolic ratios of the umbilical cord artery greater than 3.0, whereas none of the 8 BWCTs exhibited this finding (P=0.050) (Tables 1, 2). A systolic/diastolic ratio of the umbilical cord artery greater than 3.0 suggests that the fetus is receiving an insufficient amount of blood due to resistance in the placental vasculature and has been related to low birth weight and pre-eclampsia (Bruner et al., 1993; Moon, 1999).

Seven among the 26 women pregnant with BWDTs had hypertension, and five among the 7 hypertensive women pregnant with BWDTs had preeclampsia. Two of the 26 women pregnant with BWDTs had uterine leiomyomas, and had adenomyosis. None of the 10 mothers of BWCT exhibited a history of hypertension, preeclampsia, uterine leiomyoma or adenomyosis. The heterogeneous placental findings may result from local deficiencies of oxygen content. Additionally, the pre-eclampsia singleton group exhibited a higher capillary to terminal villous ratio and TV syncytial knotting relative to the normal singleton group, further suggesting placental adaptation of BWDTs in preplacental or uteroplacental hypoxia.

GLUT3 was largely expressed on the basal membranes of the syncytiotrophoblast in this study. This expression on the basal membranes of the syncytiotrophoblast is consistent with a previous study of GLUT3 expression on syncytiotrophoblasts and cytotrophoblasts in human placentas of late-onset intrauterine growth restriction (Janzen et al., 2013). The density of GLUT1 on the basal membrane of the syncytiotrophoblast and the permeability of the basal membrane are major factors in determining fetal glucose concentrations (Barta and Drugan, 2010). HIF-1α has been identified as a key transcription factor that mediates the cellular response to hypoxia. During hypoxic conditions, HIF-1α degradation is stopped, subsequently resulting in the induction of endothelial cell proliferation (Lee et al., 2004). In our study, we found that nuclear HIF-1α expression was increased in the capillary endothelial cells of TV in pre-eclampsia placentas

compared with normal singleton placentas (P<0.001). The differential HIF-1α expression in TV capillary endothelial cells between each twin pair of BWDT placentas showed a statistical significance relative to the BWCT placenta group (P=0.007). Elevated GLUT3 expression in the placentas of the smaller babies in BWDT pregnancies suggests that increased GLUT3 expression on the basal layer of the syncytial trophoblast and endothelial cells compensates for low glucose concentrations and serves to elevate oxygen and glucose concentrations. Overall, our results suggest that preplacental or uteroplacental hypoxia is more strongly associated with birth weight-discordant pregnancy than postplacental hypoxia in live neonates. To maintain placental and fetal glucose concentrations under low-oxygen conditions, differential TV proliferation and increased maternal GLUT3 expression on the terminal villous capillaries, the primary site of fetomaternal oxygen exchange, are necessary in BWDT placentas.

In conclusion, our findings indicate that the number of TV, the degree of syncytial knotting, the extent of terminal villous capillary proliferation and GLUT3 expression on trophoblasts and TV capillary endothelial cells are associated with placental adaptation of BWDT, resulting in preplacental or uteroplacental hypoxia. Given the complexity of the etiology of placental damage and fetal hypoxia, future studies will be required to ascertain the differential microscopic findings and GLUT3 expression among BWDT placenta that represent adaptive changes to hypoxia.

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