Article **Open Access**

Birth Outcomes and *IGF2* Methylation in P3 Promoter Region in Tibetan and Han Chinese Maternal-newborn Pairs in Hypobaric Hypoxia High-altitude Area

Wenxiu Jian*, Pin Sun*, Xuejun Wang*, Pinhua Wang, Jiangzuo Zhaxi, Yangrui Zhang, Shulin Wang, Dexiong Zhao, Yuemei Fan, Liehong Wang, Wen Peng[1]

January 10, 2023

DOI:10.36316/gcatr.05.0047

ABSTRACT

Background: The relationship between Insulin-like growth factor 2 (*IGF2*) methylation in the P3 promoter region and birth outcomes in a hypobaric-hypoxia environment has never been investigated. This study examined the association and compared birth outcomes and *IGF2* methylation in this region by ethnicity and altitude.
Methods: Four hundred and six (406) mother and newborn pairs in the Tibetan Plateau were enrolled in a birth cohort study. Data were collected through interviews using structural questionnaires or extracted from medical records. Pyrosequencing was performed for *IGF2* methylation in the P3 promoter region in maternal peripheral and umbilical cord blood. Birth outcomes and *IGF2* methylation were compared among three groups: Han in high altitude (HHA, n=164, 2000-3500m), Tibetan in high altitude (THA, n=42, 2000-3500m), and Tibetan in ultra-high altitude (TUHA, n=200, 3500m and higher).
Results: TUHA seemed to have a higher prevalence of macrosomia (7.5%) than both THA (0.0.%) and HHA (2.4%) and a lower *IGF2* methylation level in maternal blood than THA (*P*=0.008). No difference in the *IGF2* methylation levels was found between THA and HHA. The *IGF2* methylation levels in maternal peripheral blood were associated with a reduced risk of macrosomia (RR= 0.726, 95% CL [0.528,0.998], *P*=0.049) among all mother and newborn pairs.
Conclusions: Increased altitude appears to be associated with decreased maternal *IGF2* methylation levels in the P3 promoter region, and maternal *IGF2* methylation levels in this region was associated with reduced risk of macrosomia in newborns in the hypobaric hypoxic Tibetan Plateau environment.

KEYWORDS

Macrosomia; *IGF2;* DNA methylation; high altitude; Tibet Plateau

[1]Correspondence to: W Peng, email: wen.peng2014@foxmail.com, P Sun, email: pinsun@fudan.edu.cn, or L Wang, email:2774735671@qq.com; * authors contribute equally to this work.

INTRODUCTION

The hypobaric hypoxia environment of the high-altitude Plateau may negatively affect birth outcomes and lead to the delayed postnatal growth (1). Animal experiments have confirmed that hypoxia can cause fetal developmental restriction after adjusting for nutritional status (2). Epidemiological studies have also shown that fetal birth weight gradually decreases with elevation (3, 4), and infants of multi-generational residents seemed less likely to have potential adverse effects of high-altitude hypoxia (4). Highlanders in different geographic areas, for example, Tibetan and Andean, showed different patterns in adaptation to high-altitude hypoxia (5).

However, the above epidemiological results were obtained decades ago. Recent comparative studies of birth outcomes among populations in highlands are lacking. Further, few population studies have investigated the potential mechanisms of the impact of high altitude on birth outcomes. The seriously lagging socioeconomic status and poor prenatal care resources in high-altitude areas could be important confounding factors in adverse birth outcomes.

DNA methylation may mediate the potential negative impact of high altitude on birth outcomes. Insulin-like growth factor 2 (*IGF2*) is located on chromosome 11p15, which harbors a cluster of imprinted genes, and encodes hormones that can regulate intrauterine fetal development. In particular, *IGF2* is thought to be important in modulating fetal nutrient transport (6). Animal experimental studies have indicated an association of the *IGF2*/*H19* imprint control region (ICR) methylation with birth weight in progeny of mice (7). Epidemiological studies, despite incon-sistent results, have suggested an association between *IGF2* methylation levels and birth weight (8-10). Also, methylation at a CpG site outside *IGF2* DNA methylation regions (DMRs) impacts birth weight (11). However, few studies have investigated the potential effects of DNA methylation levels in the *IGF2* promoter region on birth outcomes, despite promoters being considered important regulators for transcription.

Human *IGF2* gene comprises five promoters (P0-P4, Fig. 1) that are spatially and temporally controlled (12, 13). The P2 promoter region is considered an important and active regulator of *IGF2* transcript but has been largely under-studied. The Genotype-Tissue Expression project (GTEx), which identified genetic variants that influence how genes are turned on and off in human tissues and organs, suggested that 76 to 99 percent of all *IGF2* mRNA expression was regulated by P3 and P4 promoters (12). Meanwhile, P2 and P3 promoters are predominantly active in both fetuses and adults (14). Therefore, the overlapping P3 promoter may be a particularly interesting target region to regulate transcription. So far, we have not found any study that explored the potential regulating role of DNA methylation levels in the *IGF2* P3 promoter region on birth outcomes.

To fill this research gap, this study was conducted using a birth cohort (mother-newborn pairs) in Han and Tibetan populations from the Tibetan Plateau. The specific aims included 1) comparing the prevalence of birth outcomes between Han and Tibetan mothers in high altitude environments; 2) examining the DNA methylation levels in the *IGF2* P3 promoter region in mothers and newborns by ethnicity (Han and Tibetan) and by living altitudes (2000-3500m and 3500m and higher); and then 3) investigating the association between *IGF2* methylation levels in the P3 promoter region in mothers/newborns and birth outcomes.

METHODS

Participants

The subjects were from a birth cohort study conducted in two major maternity hospitals in two locations from the Tibetan Plateau. One was Xining, the largest city in the Plateau, at an elevation of about 2200 meters above sea level. The other was Nangqian County in Yushu Tibetan Autonomous Prefecture, Qinghai Province, at around 3700 meters above sea level. Residents in Xining and neighboring areas are multi-ethnic groups, such as Han, Tibetan, and Hui ethnicity, while residents in Nangqian are almost all Tibetan. The environments and livelihoods in Xining and Nangqian were utterly different. The former is the capital city of Qinghai Province, with a population of 2.39 million. The latter is a remote rural Tibetan area with only 90.3 thousand population sparsely distributed in an area of 12,061 km^2. Residents in Nangqian are mainly Tibetan farmers and/or pastoralists.

The enrolled subjects were admitted to hospitals in the third trimester during hospital visits from 2019 to 2020. Written informed consent was obtained before the enrollment. The study was approved by the ethical committee of the Medical College of Qinghai University (Approval No: 2018-36). Information on participant identifications during the study was anonymized. Information on demographics, socioeconomic status, lifestyle, living and occupational environments, and related histories were collected through face-to-face interviews by well-trained investigators. Subjects' obstetric history, anthropometric measurements, and lab test results in the third trimester closest to the delivery were extracted from medical records. The lab technicians of the Nangqian hospital were trained by those in the Xining hospital. Both hospitals had certified clinical labs and consistent protocols. After the delivery, maternal information and newborn birth outcomes were extracted from medical records.

The inclusion criteria for this study were: mother and newborn pairs (1) of Han or Tibetan ethnicity of maternal original; (2) with completed questionnaires and medical records; (3) with completed maternal peripheral blood and umbilical cord blood samples for DNA methylation tests. Subjects who could not complete the interview or provide

a blood sample collection were excluded. A proportion of 86.5% newborns enrolled had both parents of the same ethnicity of Han or Tibetan.

Blood sample collection

Maternal peripheral blood and umbilical cord blood samples were collected by certified medical staff using anticoagulation tubes ($EDTAK_2$, SANLI, Liuyang, China). The blood sample was collected in the third trimester several days before the delivery or at the delivery in case of an emergency admission. All umbilical cord blood samples were collected during the delivery. The collected samples were stored in a refrigerator at 4°C until DNA extraction.

DNA extraction and Methylation analysis

DNA extraction was conducted using whole blood samples within 48 hours after collection. Genomic DNA was extracted using the TIANamp Blood DNA Kit (Tiangen, China) in accordance with the manufacturer's protocol. EZ DNA Methylation Kit (Zymo Research, USA) was used to complete the bisulfite treatment of DNA, converting unmethylated cytosine to uracil without changing methylated cytosine. Bisulfite converted DNA (500ng) was amplified by PCR in a 30 ml reaction volume using the 2×Taq PCR Master Mix with 15ul and 0.5ul each of the forward and reverse Primer. Four microliters of the post-bisulfite-treated DNA were used for PCR amplification.

The *IGF2* P3 methylation levels in maternal peripheral blood and umbilical cord blood were determined using the method, and sequences of the primers provided by Ji were used (15). Our assay amplicon is located on the genomic position of chr11:2,137,534-2,137,641(GRCh38/hg18). Figure 1 shows the map and primers position(16). The average methylation levels of maternal peripheral blood and umbilical cord blood were provided in Table 1. The genomic DNA was denatured into single-strands and then treated with sodium bisulfite, which selectively converted the unmethylated cytosines into uracils through deamination while the methylated cytosines remained unchanged. Bisulfite-converted DNA was amplified using 2×Taq PCR Master Mix (Lifefeng, China). All reactions were performed using provided PCR mixtures (total volume at 30μl) with 0.5uM each of the forward and reverse PCR primers. The reverse primer was conjugated to biotin. 5 μL amplified PCR product was electrophoresed to identify the specificity of the PCR product. Sequencing was performed only if the PCR product had no nonspecific bands or primer dimers. The PCR cycling conditions were as follows: 95°C for 15min, 35 cycles of 98°C for 10s, 55°C for 30s, 72°C for 30s, and finally 72 °C for 1min. The following primers were used, *IGF2*: Forward Primer (5' to 3'): GTTTGAGGTTAAGAAGGGTAGAGT; Reverse Primer (5' to 3'): Biotin-AAAAAAATCTCCTTCCCACCTCCTTATAT; Sequencing Primer (5' to 3'): GTTAAGAAGGGTAGAGTT. The single-stranded amplicons were isolated using the Pyrosequencing Work Station. The pyrosequencing was performed using the PyroMark Q24 system (Qiagen, Toronto, Ontario, Canada) according to the manufacturer's protocol.

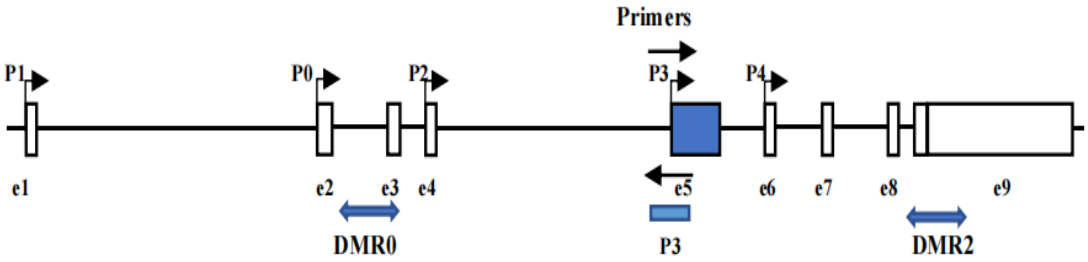

Figure 1. Structure of human *IGF2* gene with promoters (P0–P4) and region showing primer annealing used for PCR.

Table 1. The average methylation level of maternal peripheral blood and umbilical cord blood (%)

	CpG1	CpG2	CpG3	CpG4	CpG5
Umbilical cord blood	4	5	6	4	11
Maternal peripheral blood	6	7	8	6	13

The biotinylated PCR products were purified using streptavidin-Sepharose beads (Amersham, New Jersey, USA) and sequenced using the PyroMark Gold Q96 kit (Biotage AB, Uppsala, Sweden). The PyroMark Q-CpG software was applied to obtain quantitative methylation levels of targeted CpG (Cytosine-guanosine dinucleotides) sites in *IGF2*.

Outcome variables

Newborn outcomes included birth weight, length, head circumference, preterm birth (PB), low birth weight (LBW), macrosomia, and ponderal index (PI). PB was defined as a birth before gestational week 37, and term birth as birth during gestational week 37 or later. LBW was defined as a birth weight less than 2,500 g, macrosomia was defined as a birth weight of 4,000 g and greater, and others with birth weights of 2500-4000h were considered normal birth weights (NBW) (17, 18). PI was determined by weight in g / (length in cm)^3×1000. According to the gender and gestational age-specific PI percentile for Chinese newborns (19), PI between the 10th centile and the 90th centile was considered normal, PI less than the 10th centile was defined as low PI (LPI), and PI greater than the 90th centile was defined as high PI (HPI).

Covariates

Covariates were obtained from structured questionnaires and medical records. Maternal prenatal characteristics, including the status of active and passive smoking, education, and folate supplementation, were collected using questionnaires. Information on maternal age at delivery, pre-pregnancy weight and height, parity, serum albumin (g/L), diabetes mellitus, and gestational diabetes mellitus were collected from medical records. The pre-pregnancy body mass index (BMI) was calculated by dividing the pre-pregnancy weight (kg) by the pre-pregnancy height (m^2). For the data analysis, BMI was categorized into a three-category of underweight (< 18.5 kg/m^2), normal (18.5 kg/m^2 =< BMI< 24 kg/m^2) and overweight or obesity (>= 24 kg/m^2); maternal education was recoded into graduate or higher, high school or equivalent, compulsory middle school and lower, as well as never attended school; parity was divided into nulliparous and multiparous (>=1). In addition, all subjects were divided into high-altitude (2,000-3500m) residents and ultra-high altitude (3500 m and higher) residents based on long-term habitation.

Statistical analysis

Descriptive statistics were calculated for maternal or newborn demographics, socioeconomic status, and lifestyle characteristics. Mean ± SD was used for continuous variables, and n (%) was used for binary and categorical variables. The differences between the Han in high altitude (HHA), Tibetan in high altitude (THA), and Tibetan in ultra-high altitude (TUHA) were evaluated by the Kruskal-Wallis test for continuous variables and the Chi-squared test or Fisher's exact test for categorical variables, followed by Bonferroni's multiple comparison test. Multinomial logistic regression was used to obtain the risk ratios (RRs) and 95% confidence limits (CLs) of the estimates of IGF2 methylation levels in maternal peripheral blood or umbilical cord blood for HPI, LPI, LBW, macrosomia, and PB. The adjustments for covariates were performed in a hierarchical way, first for maternal age at delivery, ethnic group, infant sex (in all newborns), and gestational weeks (Model 1), then adding parity, mother's education, altitude and passive smoking (Model 2), and finally adding gestational diabetes mellitus, diabetes mellitus, folic acid use and serum albumin (Model 3). All statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary NC, USA), and $P < 0.05$ for a 2-tailed test was considered significant.

RESULTS

Four hundred and six (406) mother and newborn pairs were included in this study, with 164 Han and 242 Tibetan. All 164 pregnant Han women lived in high altitudes, and most Tibetan women (82.6%, 200/242) lived in ultra-high altitudes. During the same period, 1337 deliveries occurred at high altitudes, with 887(66.3%) of Han, 357 (26.7%) of Tibetan, and 93(7.0%) of other ethnicities; in the ultra-high altitude, 256 deliveries occurred with all Tibetan. Tibetan mothers generally had a lower socioeconomic status compared with Han. In particular, Tibetan mothers at ultra-high altitudes had a significantly lower level of education and lower rates of folic acid supplementation than the other two groups, Han and Tibetan at high altitudes (Table 2).

Birth outcomes by level of altitude and ethnicity

Newborn birth outcomes of HPI and Macrosomia also differed by level of altitude and ethnic groups. Tibetan newborns at ultra-high altitudes had significantly higher rates of HPI than those at high altitudes (15.0% vs. 0.0%, P =0.008). The Macrosomia prevalence in Tibetan newborns at ultra-high altitudes was numerically higher than in Tibetan and Han newborns at high altitudes but was statistically insignificant (7.5%, 0.0%, 2.4%, respectively, Table 2). Other birth outcomes, including PB and birth weight, were not significantly different between groups by either ethnicity or living altitude (Table 2).

IGF2 Methylation levels by altitude and ethnicity

There was a significant difference in average methylation levels among three groups in maternal peripheral blood, but no significant difference among groups in umbilical cord blood. Higher altitudes tend to be associated with lower *IGF2* methylation levels in the maternal peripheral blood sample. Compared to Tibetans living at high altitudes (2000-3500m), Tibetan residents at ultra-high altitudes (3500 m and higher) had significantly lower *IGF2* methylation levels in maternal peripheral blood (Mean 7.41±2.47 SD vs. 8.49±3.12, P = 0.008). Still, no significant difference was observed in umbilical cord blood in Tibetans between the high and ultra-high-altitude areas (Mean 5.50±1.04 SD vs. 5.84±1.60, P = 0.093). There were

Table 2. Maternal and newborn characteristics by maternal ethnic group and residential altitudes

	Total	High altitude (2000-3500m)						Ultra-high altitude (3500m and higher)			*P**
		Han (n=164)			Tibetan (n=42)			Tibetan (n=200)			
	n	*n*	*M*	*SD*	*n*	*M*	*SD*	*n*	*M*	*SD*	
Maternal factors											
Age at delivery	406	164	29.1	4.4	42	28	5.2	200	28.1	5.7	0.065
Gestational weeks	406	164	39.5	1.3	42	39.6	1.4	200	39.6	1.2	0.252
Education[1]											<0.001
Graduate or higher	170	121	75.2		29	69		20	10.5		
High school	24	19	11.8		1	2.4		4	2.1		
Middle or below	64	20	12.4		10	23.8		34	17.9		
Never school	135	1	0.6		2	4.8		132	69.5		
BMI[1]	214	161	20.9	3.1	42	20.9	2.8	11	21.7	3	0.596
Less than 18.5	48	38	23.6		9	21.4		1	9.1		0.787
18.5-	130	97	60.3		26	6.9		7	63.6		
24.0 and above-	36	26	16.1		7	16.7		3	27.3		
Parity											<0.001
Nulliparous	125	67	40.9		21	50		37	18.5		
Multiparous	281	97	59.1		21	50		163	81.5		
Passive smoking[1]											0.015
No	267	100	63.3		25	59.5		142	75.9		
Yes	120	58	36.7		17	40.5		45	24.1		
Diabetes mellitus[1]											0.007
No	382	155	95.7		42	100		185	100		
Yes	7	7	4.3		0	0		0	0		
Gestational DM[1]											<0.001
No	368	131	79.9		38	90.5		199	100		
Yes	37	33	20.1		4	9.5		0	0		
Folic acid use[1]											<0.001
No	139	7	4.3		4	9.5		128	68.8		
Yes	252	156	95.7		38	90.5		58	31.2		
Albumin(g/L)[1]	361	144	35.5	2.8	38	33.9	3.7	179	36.2	3.7	0.01
Newborn factors											
Sex											0.137
Male	207	93	56.7		18	42.9		96	48		
Female	199	71	43.3		24	57.1		104	52		
Preterm birth											0.776
No	393	159	97		40	95.2		194	97		
Yes	13	5	3		2	4.8		6	3		
Weight (g)	406	164	3197	472	42	3195	495	200	3221	513	0.112
Low	30	14	8.5		3	7.1		13	6.5		
Normal	357	146	89.1		39	92.9		172	86		
Macrosomia	19	4	2.4		0	0		15	7.5		
Body length (cm)	406	162	50.8	2.9	42	50.9	2.6	200	50.4	3	0.04
Head circumfer.[1]	401	162	32.2	2.4	41	31.8	2.4	198	34.1	2.6	<0.001
Ponderal index[1]	404	162	24.2	2.4	42	24.1	1.7	200	25.4	5.1	0.018
Low (<P_{10})	140	51	31.5		26	61.9		97	48.5		<0.001
Normal (P_{10}-P_{90})	230	107	66.1		16	38.1		73	36.5		
High (>P_{90})	34	4	2.4		0	0		30	15		

[1]For some variables, counts did not add up to total due to missing values;
*P-value for comparison between high altitude and ultra-high altitude in Tibetan only; Only albumin level was statistically significant ($p<0.05$) between Han and Tibetan at high altitudes (2000-3500m);
Weight was recoded into low (<2500 g), normal (2500-4000 g) and macrosomia (>=4000 g).
GDM, gestational diabetes mellitus; Head circumfer, head circumference (cm); BMI, pre-pregnancy BMI.
Ponderal index (kg/m^3) was recoded into a three category-variable based on percentile.
M, mean for continuous variables and proportion for categorial variables, SD standard deviation for continuous variables;
The active smoking data not shown because only 0.99% women were active smokers in our study.

no statistically significant differences in the *IGF2* methylation of maternal peripheral and umbilical cord blood between Han and Tibetan women who lived at high altitudes (Figure 2). Unfortunately, we did not have Han women living in ultra-high altitudes, although we had a large sample of Tibetan women.

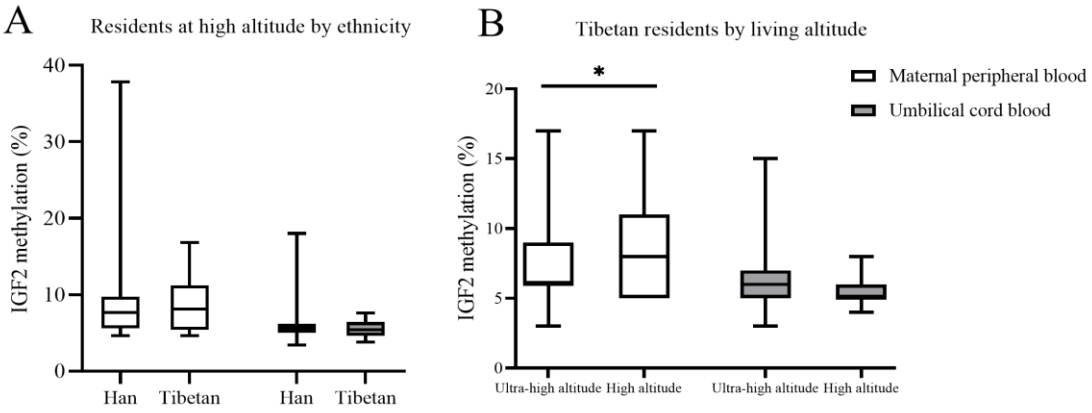

Figure 2. Mean levels of *IGF2* methylation in maternal peripheral and umbilical cord blood by ethnicity and altitude.
A. Han and Tibetan residents at High altitudes (2000-3500m). B. Tibetan residents living in High altitudes (2000-3500m) and Ultra-high altitudes (3500 m and higher).
** P<0.05*

IGF2 methylation and birth outcomes

In all newborns, *IGF2* methylation levels in maternal peripheral blood were associated with reduced risk of macrosomia (RR = 0.726, 95% CL [0.528, 0.998], *P* = 0.049) after adjustment for covariates (Model 3). The association became statistically not significant after stratification by newborn sex (males, RR = 0.515, 95% CL: [0.254, 1.044], *P* = 0.066; females, RR=0.870, 95% CL [0.590, 1.284], *P* = 0.484) (Table 3). However, there were no statistically significant associations of *IGF2* methylation levels in umbilical cord blood with birth weight after full adjustments (Supplementary Table 1). No significant association was observed between *IGF2* methylation level and other birth outcomes (data not shown).

DISCUSSIONS

Our study found Tibetan newborns living in ultra-high-altitude areas had a significantly higher ponderal index and numerically appeared to display an increased risk of macrosomia but lower *IGF2* methylation levels in the P3 promoter region in their maternal blood. The lower *IGF2* methylation levels in maternal peripheral blood suggests a potential association with an increased risk of macrosomia. The relationship between maternal DNA methylation levels of *IGF2* P3 promoter region and macrosomia may help disclose mechanisms by which a hypoxic environment affects birth outcome and provide insights into prenatal care in general populations.

This study is one of the first to examine the association of maternal and newborn DNA methylation levels in the *IGF2* P3 promoter region with birth outcomes. The unique population living in the high-altitude hypoxia environment and the Tibetan and Han mother-newborn pairs have enhanced the value of the present study in an under-studied population and a unique setting. Barriers in natural environments, infrastructure, language, and other factors in the Tibetan Plateau, especially at ultra-high-altitude levels, are considerable challenges in conducting such studies.

Environmental conditions could induce alterations of DNA methylations that may influence the development of disease. Some researchers had investigated blood methylation levels in Tibetan chickens at ultra-high altitudes (3,500 m and higher) and discovered that methylation levels in the promoter were relatively low. At the same time, the gene body was also hypomethylated (20). In line with this research, we found decreased DNA methylation levels in the *IGF2* P3 promoter region in maternal peripheral blood among those who lived in ultra-high-altitude areas, which suggested that high altitude might impact fetal growth through IGF2 promoter methylation.

Table 3. Multinomial logistic regression estimates of the association between IGF2 methylation in maternal peripheral blood and neonatal weight

	Neonatal weight status	*β*	*SE*	*RR*	*P*	95% Confidence Limit	
						Low	Upper
Overall	Model 1 [a]						
	Normal			1.000			
	Low	-0.007	0.066	0.993	0.919	0.874	1.129
	Macrosomia	-0.258	0.128	0.772	0.044	0.601	0.993
	Model 2 [b]						
	Normal			1.000			
	Low	-0.032	0.075	0.968	0.669	0.835	1.122
	Macrosomia	-0.242	0.137	0.785	0.078	0.600	1.027
	Model 3 [c]						
	Normal			1.000			
	Low	-0.017	0.086	0.983	0.842	0.831	1.163
	Macrosomia	-0.321	0.163	0.726	0.049	0.528	0.998
Male	Model 1 [a]						
	Normal			1.000			
	Low	0.110	0.085	1.117	0.192	0.946	1.318
	Macrosomia	-0.436	0.244	0.646	0.073	0.401	1.042
	Model 2 [b]						
	Normal			1.000			
	Low	0.083	0.107	1.087	0.436	0.881	1.340
	Macrosomia	-0.465	0.276	0.628	0.092	0.365	1.080
	Model 3 [c]						
	Normal			1.000			
	Low	0.122	0.111	1.130	0.271	0.909	1.404
	Macrosomia	-0.664	0.360	0.515	0.066	0.254	1.044
Female	Model 1 [a]						
	Normal			1.000			
	Low	-0.198	0.142	0.820	0.163	0.621	1.083
	Macrosomia	-0.154	0.149	0.857	0.300	0.640	1.148
	Model 2 [b]						
	Normal			1.000			
	Low	-0.299	0.185	0.742	0.107	0.516	1.066
	Macrosomia	-0.116	0.158	0.890	0.461	0.653	1.213
	Model 3 [c]						
	Normal			1.000			
	Low	-0.284	0.199	0.753	0.153	0.510	1.111
	Macrosomia	-0.139	0.198	0.870	0.484	0.590	1.284

Note: Low birth weight was defined as a birth weight less than 2,500 grams, macrosomia was defined as a birth weight of 4,000 grams and greater, and others with birth weights of 2500-4000 grams were considered normal birth weights.
a: adjusted for maternal age at delivery, ethnic group, infant sex (in all newborns), and gestational weeks.
b: Model 1 covariates plus parity, education, altitude, passive smoking.
c: Model 2 covariates plus gestational diabetes mellitus, diabetes mellitus, folic acid, albumin.

We also found that mothers' *IGF2* promoter methylation levels appear to be negatively associated with macrosomia in newborns, but the association was not significant due to limited sample size for Tibetan sample only. The association between methylation levels in *IGF2* genes and birth outcomes has been inconsistent in other population studies (21-23). Most research has focused on methylation in umbilical cord blood or placental tissue at three major differential methylation regions (DMRs), DMR0, DMR2, and the imprinting control region (ICR 1) because the expression of *IGF2* is predominantly regulated by numerous DMRs found in *IGF2* and neighboring *H19*. For instance, Liu et al. observed a lower *IGF2* methylation level in DMR in the cord blood among the LBW compared with normal-weight newborns (22). Later, Bouwland-Both et al. reported that being small for gestational age (SGA) was associated with lower *IGF2* DMR methylation (24). Some other studies have revealed the association between *IGF2* methylation levels and fetal birth weight (25, 26), though others did not (27).

Furthermore, a recent study found reduced methylation levels at some CpG sites of *IGF2* DMR in a macrosomia group compared to a normal birth weight group (28). Intriguingly, outside of established DMRs, we observed an association between macrosomia and *IGF2* promoter methylation in maternal blood. The association disappeared when stratified by newborn's sex, probably due to the limited sample size in each stratified group. Similar to our findings, a recent study identified a significant negative association between birth weight and *IGF2* methylation, which included a CpG site with a promoter region but excluded any areas inside the DMRs in maternal blood (11). These findings indicated that maternal *IGF2* methylation patterns might affect fetal growth, albeit through different mechanisms than DMR-based, established imprinting regulation pathways. These contradictory results may also be attributed to different study settings and subjects' characteristics. The high-altitude environment and the unique Tibetan population could explain the disparity. The findings from our study suggests the potential application of *IGF2* methylation as an epigenetic marker for personalized prenatal care to prevent macrosomia among high-altitude populations.

This study suggests public health implications on reproductive and maternal health. We found no significant differences in newborn birth outcomes, including birth weight and preterm birth between groups by ethnicity or altitude in this study. This supports the view that China has made massive progress in women's reproductive, maternal, and newborn health in the past decades, particularly in disadvantaged areas (29). However, some socioeconomic and behavioral factors, which may affect maternal and child health, differed significantly between Tibetan and Han Chinese mothers. Some examples are that Tibetan mothers generally had a low education level, less than half of Tibetan mothers had taken a folic acid supplement before or during pregnancy, and only around 20% knew their pre-pregnancy body weight.

Moreover, the regional disparity in reproductive and maternal health in China is also a significant public health issue (30, 31). Health equity is important in China's national health initiative Healthy China 2030. Actions are needed to remedy these disparities. Particular attention to disadvantaged populations is required, such as the Tibetan population in hypoxic high-altitude areas.

This study had several limitations. First, recall bias may exist for information collected through face-to-face interviews. Second, dietary factors, which may impact birth outcomes, were not adjusted. However, the biomarker of maternal serum albumin, which represents nutritional status, has been included in the modeling. Third, the numbers of cases with adverse birth outcomes, such as LBW and macrosomia, were small, which may increase the likelihood of false positive results. Fourth, the expression of *IGF2* mRNA was not evaluated in our study. The main reason was that one project site was located in a very remote and resource-limited area at 3700 meters high, making it impossible to have adequate infrastructure and qualified technicians in the field to manage RNA samples quickly. Fifth, the effects of methylation changes in the other regions mentioned earlier (DMR0, DMR2, and IC1) were not included. Sixth, the number of mothers and newborns in HHA, THA, and TUHA were unbalanced. Seventh, some selection bias may exist, and the participants enrolled were possibly not representative of the entire population of mother-newborn pairs in this region. However, the comparative study design is capable of answering the research question.

Future studies must examine the effects of gene-gene and gene-environment interactions on fetal growth in the high-altitude hypoxia environment.

CONCLUSION

In conclusion, increased altitude may be associated with decreased maternal *IGF2* methylation levels in the P3 promoter region, and maternal *IGF2* methylation levels in this region was associated with reduced risk of macrosomia in newborns in the hypobaric hypoxic Tibetan Plateau environment. Further studies are needed to investigate the gene-environment interactions and potential adaptation mechanisms in high-altitude areas. Actions are necessary to promote maternal education and prenatal care among Tibetans to achieve health equity.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

AUTHOR CONTRIBUTIONs

Methodology, investigation, and manuscript writing, (WJ); methodology, formal analysis, resources, and manuscript writing (PS); investigation and resources (XW, PW, ZXJC, YZ, DZ, LW); investigation and supervision (SW); review the

manuscript (YF); study design, funding acquisition, methodology, investigation and manuscript writing (WP).

FUNDING

The study was funded by the Natural Science Foundation in Qinghai (grant number 2019-ZJ-932Q) and the National Science Foundation of China (NSFC) (grant number 81860579). This work was also partly supported by the Danone Nutrition Center (grant number DIC 2018-08) and the Medical College of Qinghai University (foundation grant number 2019-kyt-02). The funders had no role in study design, data collection, and analysis, publication decisions, or manuscript preparation.

INSTITUTE REVIEW BOARD STATEMENT

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the ethical committee of the Medical College of Qinghai University (Approval No: 2018-36).

INFORMATED CONSENT STATEMENT

Informed consent was obtained from all subjects involved in the study.

ACKNOWLEDGEMENT

We thank Professor Yunhui Zhang and graduate students Jiahao Yang, Hang Wang, and Liyi Zhang from Fudan University for their help in experiments. Thank Guolian Qi, Min He, Jinxue Wang, Runhua Li from Qinghai University, and all of the medical care personnel in the obstetrics department of Qinghai Red Cross Hospital and Nangqian People's Hospital for their help and support of this study.

DATA AVAILABILITY

The data supporting this study's findings are available in the manuscript. The original data are not publicly available, restricted by the genetic resource regulations in China.

ARTICLE INFORMATION

Received December 12, 2022; accepted December 30, 2022; published Jan 15, 2023

DOI:10.36316/gcatr.05.01

Author Information

Nutrition and Health Promotion Center, and Department of Public Health, Medical College, Qinghai University, Xining (WJ, YZ, SW, WP); Department of Occupational Health and Toxicology, School of Public Health, Fudan University, Shanghai (PS); Department of Anesthesiology, Qinghai Red Cross Hospital, Xining, Qinghai, China (XW); Department of Obstetrics, Qinghai Red Cross Hospital, Xining, Qinghai, China (PW, DZ); Nangqian People's Hospital, Yushu, China (JZ); Center for Child, Adolescent and Maternal Health Research, Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland (YF); Qinghai Provincial Key Laboratory of Traditional Chinese Medicine Research for Glucolipid Metabolic Diseases, Medical College, Qinghai University, Qinghai, China (WP).

REFERENCES

1. Mortola JP, Frappell PB, Aguero L, Armstrong K. Birth weight and altitude: a study in Peruvian communities. J Pediatr. 2000;136(3):324-9.
2. Giussani DA, Salinas CE, Villena M, Blanco CE. The role of oxygen in prenatal growth: studies in the chick embryo. The Journal of Physiology. 2007;585(3):911-7.
3. Moore LG, Charles SM, Julian CG. Humans at high altitude: hypoxia and fetal growth. Respir Physiol Neurobiol. 2011; 178(1):181-90.
4. Soria R, Julian CG, Vargas E, Moore LG, Giussani DA. Graduated effects of high-altitude hypoxia and highland ancestry on birth size. Pediatr Res. 2013;74(6):633-8.
5. Beall CM. Tibetan and Andean contrasts in adaptation to high-altitude hypoxia. Adv Exp Med Biol. 2000;475:63-74.
6. Reik W, Constância M, Fowden A, Anderson N, Dean W, Ferguson-Smith A, et al. Regulation of supply and demand for maternal nutrients in mammals by imprinted genes. J Physiol. 2003;547(Pt 1):35-44.
7. Shao W-J, Tao L-Y, Gao C, Xie J-Y, Zhao R-Q. Alterations in methylation and expression levels of imprinted genes H19 and Igf2 in the fetuses of diabetic mice. Comp Med. 2008; 58(4):341-6.
8. Tabano S, Colapietro P, Cetin I, Grati FR, Zanutto S, Mandò C, et al. Epigenetic modulation of the IGF2/H19 imprinted domain in human embryonic and extra-embryonic compartments and its possible role in fetal growth restriction. Epigenetics. 2010;5(4):313-24.
9. Tobi EW, Heijmans BT, Kremer D, Putter H, Delemarre-van de Waal HA, Finken MJ, et al. DNA methylation of IGF2, GNASAS, INSIGF and LEP and being born small for gestational age. Epigenetics. 2011;6(2):171-6.
10. Liu FD, Xue G, Wang G. Relationship between methylation status of IGF2 in parturient women and neonates and birth outcomes of neonates. Maternal Child Health Care of China. 2011. Available
11. Montoya-Williams D, Quinlan J, Clukay C, Rodney NC, Kertes DA, Mulligan CJ. Associations between maternal prenatal stress, methylation changes in IGF1 and IGF2, and birth weight. J Dev Orig Health Dis. 2018;9(2):215-22.
12. Rotwein P. The complex genetics of human insulin-like growth factor 2 are not reflected in public databases. J Biol Chem. 2018;293(12):4324-33.
13. Yamaguchi Y, Tayama C, Tomikawa J, Akaishi R, Kamura H, Matsuoka K, et al. Placenta-specific epimutation at H19-DMR among common pregnancy complications: its frequency and effect on the expression patterns of H19 and IGF2. Clin Epigenetics. 2019;11(1):113.
14. Engström W, Shokrai A, Otte K, Granérus M, Gessbo A, Bierke P, et al. Transcriptional regulation and biological significance of the insulin like growth factor II gene. Cell Prolif. 1998;31(5-6):173-89.
15. Ji M, Wang X, Wu W, Guan Y, Liu J, Wang J, et al. ART manipulation after controlled ovarian stimulation may not

increase the risk of abnormal expression and DNA methylation at some CpG sites of H19,IGF2 and SNRPN in foetuses: a pilot study. Reprod Biol Endocrinol. 2018; 16(1):63.
16. Watanabe H, Higashimoto K, Miyake N, Morita S, Horii T, Kimura M, et al. DNA methylation analysis of multiple imprinted DMRs in Sotos syndrome reveals IGF2-DMR0 as a DNA methylation-dependent, P0 promoter-specific enhancer. Faseb j. 2020;34(1):960-73.
17. Cui D, Yang W, Shao P, Li J, Wang P, Leng J, et al. Interactions between Prepregnancy Overweight and Passive Smoking for Macrosomia and Large for Gestational Age in Chinese Pregnant Women. Obes Facts. 2021;14(5):520-30.
18. Ye J, Torloni MR, Ota E, Jayaratne K, Pileggi-Castro C, Ortiz-Panozo E, et al. Searching for the definition of macrosomia through an outcome-based approach in low- and middle-income countries: a secondary analysis of the WHO Global Survey in Africa, Asia and Latin America. BMC Pregnancy Childbirth. 2015;15:324.
19. Zong XN, Li H, Zhang YQ, Wu HH. Reference values and growth curves of weight/length, body mass index, and ponderal index of Chinese newborns of different gestational ages. Zhonghua Er Ke Za Zhi. 2021;59(3):181-8.
20. Zhang Z, Du H, Bai L, Yang C, Li Q, Li X, et al. Whole genome bisulfite sequencing reveals unique adaptations to high-altitude environments in Tibetan chickens. PLoS One. 2018;13(3):e0193597.
21. Bouwland-Both MI, van Mil NH, Stolk L, Eilers PH, Verbiest MM, Heijmans BT, et al. DNA methylation of IGF2DMR and H19 is associated with fetal and infant growth: the generation R study. PLoS One. 2013;8(12):e81731.
22. Liu Y, Murphy SK, Murtha AP, Fuemmeler BF, Schildkraut J, Huang Z, et al. Depression in pregnancy, infant birth weight and DNA methylation of imprint regulatory elements. Epigenetics. 2012;7(7):735-46.
23. St-Pierre J, Hivert MF, Perron P, Poirier P, Guay SP, Brisson D, et al. IGF2 DNA methylation is a modulator of newborn's fetal growth and development. Epigenetics. 2012;7(10): 1125-32.
24. Bouwland-Both MI, Van M, Lisette S, Eilers P, Verbiest M, Heijmans BT, et al. DNA Methylation of IGF2DMR and H19 Is Associated with Fetal and Infant Growth: The Generation R Study. Plos One. 2013;8(12):1389-92.
25. St-Pierre, Hivert, M.-F., Perron, ChicoutimiHospital, Poirier, et al. IGF2 DNA methylation is a modulator of newborn's fetal growth and development. Epigenetics: official journal of the DNA Methylation Society. 2012.
26. Haggarty P, Hoad G, Horgan GW, Campbell DM. DNA methyltransferase candidate polymorphisms, imprinting methylation, and birth outcome. PLoS One. 2013;8(7): e68896.
27. Burris HH, Braun JM, Byun HM, Tarantini L, Mercado A, Wright RJ, et al. Association between birth weight and DNA methylation of IGF2, glucocorticoid receptor and repetitive elements LINE-1 and Alu. Epigenomics. 2013;5(3):271-81.
28. Su R, Wang C, Feng H, Lin L, Liu X, Wei Y, et al. Alteration in Expression and Methylation of IGF2/H19 in Placenta and Umbilical Cord Blood Are Associated with Macrosomia Exposed to Intrauterine Hyperglycemia. PLoS One. 2016;11(2):e0148399.
29. Qiao J, Wang Y, Li X, Jiang F, Zhang Y, Ma J, et al. A Lancet Commission on 70 years of women's reproductive, maternal, newborn, child, and adolescent health in China. The Lancet. 2021;397(10293):2497-536.
30. Wang W, Liu F, Zhang Z, Zhang Y, Fan X, Liu R, et al. The Growth Pattern of Tibetan Infants at High Altitudes: a Cohort Study in Rural Tibet region. Sci Rep. 2016;6:34506.
31. Moucheraud C, Gyal L, Gyaltsen K, Tsering L, Narasimhan S, Gipson J. Maternal Health Behaviors and Outcomes in a Nomadic Tibetan Population. Matern Child Health J. 2018;22(2):264-73.

How to cite this article:

Jian W, Sun P, Wang X, Wang P, Zhaxi J, Zhang Y, Wang S, Zhao D, Fan Y, Wang L, Peng W. Birth outcomes and *IGF2* methylation in P3 promoter region in Tibetan and Han Chinese Maternal-Newborn Pairs in hypobaric hypoxia high-altitude area. Glob Clin Transl Res. 2023; 5(1): 1-11. DOI:10.36316/gcatr.05.0047

Supplementary Table 1. Association between IGF2 methylation in umbilical cord blood and neonatal weight

	Neonatal weight status	*β*	*SE*	*RR*	*P*	95% Confidence Limit	
						Low	Upper
Overall	**Model 1** [a]						
	Normal			1.000			
	Low	0.004	0.160	1.004	0.978	0.734	1.373
	Macrosomia	0.177	0.109	1.194	0.104	0.964	1.479
	Model 2 [b]						
	Normal			1.000			
	Low	0.026	0.168	1.026	0.877	0.738	1.427
	Macrosomia	0.164	0.111	1.178	0.140	0.948	1.464
	Model 3 [c]						
	Normal			1.000			
	Low	-0.022	0.175	0.978	0.899	0.694	1.379
	Macrosomia	0.165	0.117	1.180	0.156	0.939	1.482
Male	**Model 1** [a]						
	Normal			1.000			
	Low	-0.046	0.214	0.956	0.832	0.628	1.454
	Macrosomia	0.018	0.204	1.018	0.931	0.682	1.519
	Model 2 [b]						
	Normal			1.000			
	Low	-0.063	0.247	0.939	0.798	0.579	1.523
	Macrosomia	-0.014	0.215	0.986	0.949	0.647	1.504
	Model 3 [c]						
	Normal			1.000			
	Low	-0.049	0.237	0.953	0.838	0.598	1.516
	Macrosomia	0.102	0.198	1.107	0.607	0.751	1.632
Female	**Model 1** [a]						
	Normal			1.000			
	Low	0.028	0.275	1.029	0.918	0.600	1.764
	Macrosomia	0.349	0.175	1.418	0.045	1.007	1.997
	Model 2 [b]						
	Normal			1.000			
	Low	0.044	0.300	1.045	0.882	0.580	1.883
	Macrosomia	0.385	0.204	1.470	0.059	0.985	2.194
	Model 3 [c]						
	Normal			1.000			
	Low	-0.004	0.327	0.996	0.991	0.525	1.891
	Macrosomia	0.411	0.247	1.508	0.096	0.930	2.446

Note:
a: Adjusted for maternal age at delivery, ethnic group, infant sex (in all newborns), gestational weeks.
b: Model1 and parity, education, altitude, passive smoking.
c: Model 2 and gestational diabetes mellitus, diabetes mellitus, folic acid, albumin.