Identification of Methylation Status of IGF2/ H19 genes and Cell Blood Count among Neonates with Low Birth Weight

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ABSTRACT

Introduction: Notably, Insulin-like growth factor 2 and H19 genes are two of the imprinted candidate genes that are involved in fetal growth and development. Accordingly, alteration in DNA methylation of imprinted genes and haematological parameters has been repeatedly associated with low birth weight through intrauterine growth retardation. The mechanisms underlying this association are unknown, although epigenetic mechanisms are hypothesized; so, this was conducted to evaluate the association between alteration in DNA methylation of imprinted genes and haematological parameters and birth weight. **Methods:** This is a prospective cross sectional study conducted in White Nile State (Sudan). The birth weights (BW) of the newborns were recorded soon after delivery. Then 29 cord blood samples were randomly selected from neonates with low birth weight as cases and 44 cord blood samples from neonates with normal birth weight as controls and subsequently tested for IGF2/H19 methylation status in imprinting control region (ICR) and haematological parameters via Methylation Specific PCR (MSP) and Auto hematological Analyzer (Mindary 3000) respectively. **Results:** Our results showed no differences between cases and control in imprinting control region (ICR), that, the IGF2 and H19 imprinting genes were seen in unmethylation status in imprinting control region (ICR) of two groups. Although, there

are no significance differences in haematological parameters between cases and control group in relation to birth weight. **Conclusion:**Our findings revealed that the DNA methylation of imprinted genes and haematological parameters are not influence by reduction in neonatal birth weight. The IGF2 and H19 imprinting genes were seen in unmethylated status, indicating that the two genes remain actively in the imprinting control area and are unaffected by reduction in birth weight.

Keywords: Cord, epigenetics, imprinting, , placenta, methylation.

INTRODUCTION:

Low birth weight (LBW) is one of the major causes of many complication during perinatal period leading to infant morbidity, and mortality, as well as the risk of developmental disabilities and illnesses in future lives [1] Globally, more than 20 million newborns (an estimated 15.5% of all births) are born low birth weight each year. More than 95% of these LBW neonates are born in low- and middle-income countries (LMICs) [2]. In developed countries, intrauterine growth restriction (IUGR) is commonly associated with underlying placental insufficiency that prevents the fetus from achieving its full growth potential [3]. Recently, some growing evidence showed that placental function and fetal growth is controlled by imprinted genes during intrauterine life [5]. Not surprisingly, epigenetic disruption of imprinted genes due to early exposure to adverse environment was proposed to be associated with enhanced susceptibility to intrauterine growth retardation in addition to adult chronic diseases [5]. Importantly, genomic imprinting is an epigenetic phenomenon that ensures the parental-specific expression of subset genes is required for normal mammalian development. However, loss of imprinting represents one of the most common genetic abnormalities in growth disorders and cancer [6]. Genes that are imprinted are expressed only from one parental allele. But, a loss of imprinting result in two possible scenarios; the affected individual either has both alleles dually expressed due to activation of silent allele or no expression due to suppression of normally active allele [7]. It has been well demonstrated that prenatal exposures to adverse social or

nutritional environments result in aberrant *IGF2* methylation that could lead to deregulated expression, including loss of imprinting (LOI) or activation of expression from the normally silent maternal allele [8]. Additionally, Pliushch and his colleague found that, the hypermethylation of multiple imprinted genes associated with spontaneous abortions and stillbirths, suggesting methylation alterations may play an integral role in pregnancy loss [9]. Moreover, other studies have reported strong association between LBW and anemia, iron deficiency and low hemoglobin levels [10]. Additionally, recent studies indicated children born with a low birth weight or SGA were positively associated with the risk of anemia, possibly due to their limited iron stores, which are proportional to body weight [11].

In Sudan, in recent year we are seen an increased risk of delivering low birth weight babies, abortion, stillbirth and maternal anaemia [12]. However, the underlying molecular mechanisms linking intrauterine growth restriction and low birth weight have been poorly documented. Accordingly, there is significant interest in better understanding the molecular mechanism behind the low birth weight via investigating DNA methylation of imprinting genes (IGF2 and H19) and haematological parameters of umbilical cord blood of neonates born with low birth weight.

MATERIALS AND METHODS

General characteristics of subjects

This is a prospective cross sectional study was conducted in Eldweim teaching hospital, White Nile State, Sudan**.** Characteristics of the study subjects (n = 73 neonates) are enrolled in the study. Of 73 participants twenty nine of neonates with low birth weight and forty four with normal weight are randomly selected for studying DNA methylation and haematological parameters.

Collection of cord blood samples

Blood samples were collected from neonatal cord blood by inserting needle into the umbilical vein above the clamp and two milliliters of cord blood was collected into K_2EDTA at the time of delivery for investigating DNA

methylation of imprinting genes and haematological paramrters.

DNA Methylation Analysis

DNA was extracted from cord blood according to G-spin ™ Total DNA Extraction Kit manufacturer's recommended protocol. Then the DNA quantity and quality were assessed using a Nanodrop 1000 spectrophotometer. Then the extracted DNA subsequently underwent bisulfite treated using the EZ DNA Methylation™ Kit (Zymo Research, CA, USA) according to the manufacturer's protocol. Then, bisulfate treated DNA and design primers were added to hot start polymerase (DSBIO).

Primer Design for MSP:

The primers "M-for" and "M-rev" were designed for the methylated sequence, whereas the primers "U-for" and "U-rev" were designed for the unmethylated sequence. Every sample was tested twice for each primer.

Primers sequence:

M-for: 5'-TTAATTGGGGTTCGTTCG-3'

M- rev: 5'-CCCGACCTAAAAATCTAATACGA-3'

U-for: 5'-GGTTTGTTTGTGGAAATGTTTT-3'

U-rev: 5'-CCCAACCTAAAAATCTAATACAA-3'

Molecular detection by Methylation-specific PCR (MS-PCR)

Bisulfite-treated DNA(2 µL) was added to a 25-µL PCR reaction containing 12 µL of hot start polymerase (DSBIO), 1 µL of each of the corresponding MSP primers (10 pmol), 9µL of water for injection. Reaction mixtures were thermal cycled (methylated allele: 95 °C for 45 s, 55 °C for 30 s, and 72 °C for 30s; unmethylated allele: 95 °C for 45 s, 55°C for 30 s, and 72 °C for 30 s) for 35 cycles with an initial denaturing step of 5 min at 95 °C, 72 °C for 5 min for extension and 4 °C for 1min for cooling. Performed on MJ RESEARCH, ICN-200 Peltier Cycle/Germany and TECHNE, Prime/England.

Agarose Gel Electrophoresis

Methylation Specific PCR products were analyzed by electrophoresis on 2% agarose gels and stained with ethidium bromide. Bands were visualized under UV illuminator and photographed.

Haematological Paramrters Assesment

Haematological parameters were analyzed using Auto hematological Analizer (Mindary 3000).

Statistical analysis

Data of the study were expressed as mean ± standard deviation and further analyzed using the SPSS version 20 **(**Independent Samples Test). A P- value of P< 0.05 was used as indicator of statistical significance.

RESULTS:

The birth weights (BW) of the 73 newborns were recorded soon after delivery. Then 73 cord blood samples were randomly selected (29 from baby with low birth weight and 44 from baby with normal weight) and subsequently tested for IGF2/H19 ICR methylation status, and haematological parameters, each sample was tested twice for methylated and unmethylation sequence by methylation-specific PCR (MSP).Our results showed that all IGF2/H19 regions in 73 neonatal cord blood were positive for unmethylation, however, no methylation of IGF2/H19 region was observed among all 73 cord blood samples, as shown in figure (1). Although, table 1 showed haematological parameters results.

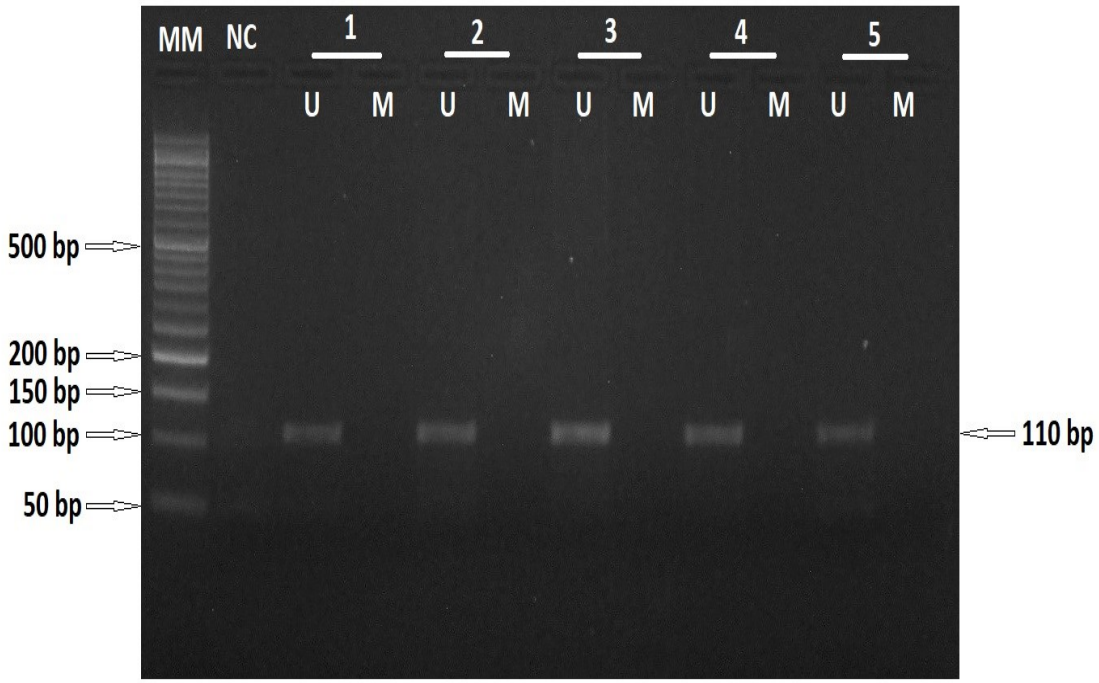


Figure 1. PCR Results of methylation status of IGF2/H19 imprinting control region in neonatal cord blood. Lane NC is control negative for IGF2/H19 ICR. Lane 1U, 2U, 3U, 4U, 5U showed clear band (110bp) is visualized PCR positive unmethylation for IGF2/H19 ICR

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No band seen in Lane 1M, 2M, 3M, 4M, 5M visualized PCR negative methylation for IGF2/H19 ICR.M: 50 bp DNA size markers on 2% agarose gel.

Table 1:Demonstrate Mean ± standard deviation of RBCs count, RBCs indices and haemoglobin types among neonates with low and normal birth weight.

Parameters	weight groups	N	Mean	Std. Deviation	P.value
RBC $^{9}10/L$	case	29	4.2076	.87657	.521
	control	43	4.1074	.42843	
PCV Pg	case	29	44.4966	4.81170	.924
	control	43	44.6023	4.42512	
MCV fl	case	29	103.8414	19.63591	.139
	Control	43	113.4651	30.57015	
MCHC g/dl	case	29	32.1276	.92655	.662
	control	43	32.0372	.80918	
Hb g/dl	case	29	14.3000	1.50000	.979
	control	43	14.2907	1.39110	
Hb F %	case	29	73.3103	8.15012	.253
	control	43	75.4000	7.12674	
Hb A %	case	29	26.0034	7.35427	.273
	control	43	24.0860	7.12302	

Case's weight lower than 2.5kg

Control's weight above 2.5 kg

Table 2: Demonstrate Mean ± standard deviation of platelets count, WBCs count and birth weight among neonates with low and normal birth weight.

Parameters	weight groups	N	Mean	Std. Deviation	P.value
Platelets $^{9}10/L$	case	29	267.4138	71.01384	.060
	control	43	233.1884	76.64207	
WBCs $^{9}10/L$	case	29	11.0172	2.31549	.394
	control	43	11.7326	4.05807	
weight (kg)	case	29	2.1759	.25587	.000
	control	43	3.0584	.45469	

Case's weight lower than 2.5kg

Control's weight above 2.5 kg

DISCUSSION:

Intrauterine growth retardation (IUGR) babies not only have immediate medical problems requiring extensive stay in neonatal units but also, are at increased risk of common comorbidities later in life; including hypertension, type 2-diabetes, and heart disease through the effects of fetal programming [3]. Events during this critical window the intrauterine life are believed to play an important role in the fetal development and growth and in determining children's susceptibility to energy metabolism-related diseases over the long-term [13]. Moreover, during intrauterine life imprinted genes play an important role in embryonic growth and development as well as in placental function [14]. In a related study, methylation and expression analysis of the H19/IGF2 locus in IUGR identified lower methylation and increased expression of H19 and decreased expression of IGF2 in disease relative to control placentas [15]. Furthermore, LOI was identified to occur in intrauterine growth retardation (IUGR) at the same rate as normal placentas, but the subset of imprinted genes showing LOI differed [16]. Interestingly, Montoya and colleagues noted that, a links between

the maternal epigenome and low birth weights rely on mechanisms outside known imprinting pathways. It thus may be important to consider the effect of maternal exposures and epigenetic profiles on birth weight even in the setting of maternally imprinted genes such as IGF2 [17]. Accordingly, the study has several strengths, including try to evaluate an association between neonatal growth retardation and haematoogical parameters abnormalities and recognitions underline molecular mechanism of association via investigating infant methylation status of imprinted genes at IGF2/H19 regions.

Intriguingly, the current results showed that, the *IGF2* and H19 imprinting genes were positive for unmethylation in imprinting control region (ICR) of neonatal cord blood, however, no methylation of *IGF2*/*H19* imprinting genes was observed among all 73 cord blood samples examined event during low birth neonates. These findings could help to explain the previously reported by Mansell and his associates, 2016 they, noted that, the IGF2 protein bind to insulin-like growth factor1 receptor (encoded by IGF1R), akinase activator that promotes cell survival and proliferation signals and consequently promotes fetal growth [18].

Additionally, the current results are in contrast with previous studies done by Ollkainen and colleagues, 2010 they, reported a week positive correlation between birth weight and IGF2/H19 ICR methylation in twins. Moreover, the data presented in this study suggested that fetal imprinting genes in IGF2/H19 imprinting control region remained actively, and not influenced by birth weight [19].

CONCLUSION:

Our findings revealed that the DNA methylation of imprinted genes and haematological parameters are not influence by reduction in neonatal birth weight. The IGF2 and H19 imprinting genes were seen in unmethylated status, indicating that the two genes remain actively in the imprinting control area and are unaffected by birth weight.

RECOMMENDATIONS:

We recommended that this study may be taken as base for carrying further study including all nine imprinting genes to verify if there is association or no between aberrant of imprinting genes

function and disturbance of haematological parameters which may later effects neonatal development**.**

CONSENT AND ETHICAL APPROVAL

Permission and informed consent was taken in advance from mothers and their husbands for participating of their babies in the study. The study received ethical approval from the Ethical committee at the Faculty of Medicine, University of Khartoum, Sudan.

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CONFLICT OF INTEREST:

We have no conflict of interest related to this work

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