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The Atypical Expression of Retinoic Acid Inducible GATA6 Protein in Placenta is a Convenient Biomarker for Newborn Health Assessment

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Abstract **Background**: Embryonic health assessment prior to delivery is not a priority in Africa due to the lack of efficient platforms for biomarker screening of defective heart or metabolic syndromes. Beside genetic mutations, some environmental, nutritional, or epigenetic events can induce abnormal protein expression impacting embryonic heart and gut developments. Among these proteins is the retinoic acid (vitamin A) inducible GATA6 which acts as transcription factor targeting the promoter of gene stimulated during placenta, embryonic heart and gut lineage specification. The objective of this study is to investigate GATA6 expression profile in placenta cells, to determine the impact of its abnormal expression on the newborn survival. **Methods**: Ethical approval of CER-ISBA) was obtained prior to placenta sample collection in the hospital obstetric service. Micro-fragments of placenta tissues (n=80) were collected after delivery and lysed for GATA6 analysis with immunoblot (western blot) method. **Results**: We observed two isoforms of GATA6 (long L and short S isoforms). All placenta lysates of living newborn expressed the type S isoform of GATA6 (n=76). In all 80 samples there is variable expression frequency for the type L isoform of GATA6. Normal expression of Type L isoform of GATA6 was observed in 63.8% of the samples; overexpression was observed in 7.5% of the samples; low expression was in 20% of them and totally lost in 8.7% of the samples. Retrospective analysis of 6 stillborn infant charts, linked 4 of them to deficient placental GATA6. **Conclusion**: Our preliminary data suggested that GATA6 could be used as biomarker for embryonic and newborn survival prognostic as well as for the postnatal screening of the risk to develop congenital heart diseases and metabolic syndromes during lifespan.

Keywords: *GATA6 isoform L, biomarker, congenital deficiency, heart defect, metabolic syndrome, stillborn*

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1. Introduction

The periconceptional environment, particularly during oocyte maturation and preimplantation development, influences the gestation model leading to permanent changes in offspring growth, physiology, health and the risk of disease until adulthood [1]. Factors such as the quality and composition of the maternal or paternal diet, parents' metabolism and health, or specific conditions used for assisted conception, such as embryo culture, can all influence the developmental program [2]. This sensitive window in the life cycle around conception can be seen in the broader context of the concept of Developmental Origins of Health and Disease (DOHD) as previously reported [3,4]. This suggests that the risk of adult disease may arise from in utero conditions where the availability of nutrients can not only control fetal growth and metabolic homeostasis, but also predispose to certain pathologies (diseases) in adults, including cardiovascular dysfunction and metabolic syndrome, if the homeostatic changes do not correspond to the postnatal environment [4]. Epidemiological studies on human populations and various animal models show support for the DOHD concept [5].

Numerous empirical studies in mice have shown that the GATA6 gene encoding for the GATA6 protein which is a transcription factor that acts by binding to the -GATA- sequences present on the promoters or first exon of target genes, to induce their transcription into messenger RNA and further their translation into proteins essential for organogenesis [6,7]. Spontaneous loss of expression of GATA6 protein in women uterine epithelial cell was

reported as molecular mechanism leading to cell dedifferention prior to cervical cancer [8]. Hence, GATA6 has a main role in the activation of epithelial cells differentiation genes and has also essential role in cardiac, hepatic, visceral, and gynecological organogenesis [6,7,8]. Six GATA transcription factors were known to intervene in organogenesis [6,7]. Among them, GATA6 function in synergy with GATA4; under specified conditions GATA4 can replace GATA6 except for cardiac functions [9]. In embryonic stem cells GATA6 expression is stimulated by all-trans-retinoic acid (Vitamin A) as well as GATA4 [10,11], GATA6 is expressed in trophectoderm cells which will give subsequently the placenta [12]. It was shown that maternal diet poor in vitamin A could affect GATA6 induction in the pre-implanting murine embryo [10,11]. In humans, mutations in GATA6 gene give isoforms of GATA6 protein and are associated with several birth defects such as congenital heart disease, metabolic syndromes, congenital diaphragmatic hernia, pancreatic agenesis, neurocognitive abnormalities, and related anomalies [13,14,15].

Furthermore, thirty-four mutations of GATA6 have been identified, but most of these mutations have been found in the adult population [13,15]. Since the placenta is the primary source of nutrients for fetal growth and development, it can be an important factor contributing to intrauterine programming induced by changes in food composition during pregnancy. Western blot data showed that GATA6 expression in embryonic cells gives two isoforms, with one long form $GATA6_L$ (60-64 KDa) and one short form $GATA6_S$ (50-52 KDa) in the same tissue [10,11].

Any genotypic or phenotypic alteration of GATA6 protein in-utero could precisely predict the risk of developing diseases in adults [16,17]. Spontaneous loss of GATA6 observed in women uterine epithelial cells leads to secondary anomaly in nuclear envelop proteins and atypia nuclear morphology [18]. It is therefore necessary to analyze the expression profile of GATA6 protein in human placenta that links the maternal uterus blood stream to the embryo. The placenta derives from trophectoderm cells and plays several roles in the development, growth, and maturation of the future embryo [16,17]. We investigated the expression pattern of GATA6 in the placenta of newborns just after birth. Accessing GATA6 expression in placenta will help to understand the cause of sudden infant death and will contribute to the prevention of cardiopathies and metabolic syndromes such as diabetes, hepatic diseases, and dyslipidemia. Our study falls within the framework of mother and child health monitoring for disease prevention.

2. Materials and Methods

2.1. Study Setting

- The University Hospital Center of SURU-LERE, Department of Motherhood and Gynecology-Obstetrics

- The Laboratory of Biomarkers in Cancerology and Nutrition (BMCN) of the Unit of Biochemistry and Molecular Biology (UBBM) of the Faculty of Sciences and Techniques (FAST) / University of Abomey Calavi (UAC) and the Institute of Biomedical Sciences and Applications (ISBA).

2.2. Materials

Biological samples: Placental samples from 100 pregnant participants aged 18-49 years. This study obtained the approval of the Research Ethics Committee of the Benin Institute of Applied Biomedical Sciences (CER-ISBA) before sample collection. All reagents for western blot were from Sigma-Aldrich and Bio-Rad. Rabbit Anti-GATA6 is custom antibody [7,8]. As loading control anti-β-actin produced in mouse was used (Sigma-Aldrich, USA). Peroxidase-conjugated secondary antibody anti-rabbit or anti-mouse and luminol reagents (Bio-Rad, USA) were used [8]. Protein standards for molecular size determination is from Bio-Rad (USA).

2.3. Methods

- Sample collection: sampling was carried out by the obstetrician-gynecologist or midwife who cut micro fragment of placenta tissue sample (weighing 1 mg) after delivery in the delivery room. The placental fragments are placed individually in sterile 5 ml tubes containing 1 ml of ice-cold Phosphate Buffered Saline (PBS) to preserve the micro-tissues during their transport to the laboratory in a cooler. The tube containing micro-tissues is stored at 4°C until processed within an hour after delivery.

- Sample processing: Tissues were washed with ice cold PBS and protein lysates were prepared for each sample by adding 200 µl of RIPA buffer to 2 mg of placental micro-tissue in Eppendorf tube. After RIPA buffer was added, tubes were incubated at 4°C for 2 h to allow cell membrane lysis and the release of proteins [8]. Then 80 µl of a denaturation solution containing tris-glycerol and SDS 5X were added before heating at 95°C for 10 minutes for proteins denaturation follows by samples storage at -20°C for subsequent analyzes [8].

- Sample analysis: In this study, western blot (immune blot) was used to analyze the expression of the GATA6 proteins. The samples are migrated on a 10% or 12 % (to visualize smaller isoforms) SDS-PAGE gel and proteins were transferred to a nitrocellulose membrane. This membrane was then incubated in a blocking solution (composed of 5% skimmed milk diluted in Tris Buffered Saline Tween-Twenty or TBST) at room temperature for 30 minutes. Immunodetection was achieved by incubating the membrane successively, in a solution of primary antibody anti-GATA6 or anti-actin (diluted in 1% milk/TBST) for 2 hours followed by 3 washes (10 minutes each) with TBST. This first immune incubation is followed by an incubation in a solution of secondary antibody anti-rabbit or anti-mouse conjugated with peroxidase (diluted in 1% milk/TBST) at room temperature for one hour. The membrane was washed 4 times (10 min each). Antibody-protein complexes were revealed by chemiluminescence on autoradiography films using an automatic processor (HQ X-ray Film Processor). The presence of GATA6 protein and isoforms appears as dark bands on the films; wild type GATA6 is at 50-55 kDa size range determined by protein standards, while actin (α or β) appears around 40-48 KDa. [8]

3. Results

3.1. Expression Profile of Transcription Factor GATA-6 in Human Placental Tissues

We observed a variation of $GATA6_L$ expression more frequently than that of $GATA6_S$ which seems to be always expressed. The presence of a dark intense band indicates a strong expression of GATA6, light dark band indicates a normal expression, very slim band indicates a weak expression whereas the absence of dark band indicates loss of GATA6 expression. The western blot images shown in Figure 1 and Figure 2 displayed samples expressing $GATA6_L$ and $GATA6_S$ as well as samples lacking $GATA6_L$ or $GATA6_S$. In some cases, isoforms of GATA6 were observed (Figure 3) indicating the presence of polymorphic mutations within the GATA6 gene. It is known that $GATA6_L$ has more activity in the genesis of cardiomyocytes and therefore its absence may underlie congenital cardiac pathogenesis. Thus, the different variations of L-type expressions and their implication will be the topic of interest in this study. Sample 3H and 3Z have lost the expression $GATA6_L$ and $GATA6_S$, while 6H has lost $GATA6_L$ but still expressing $GATA6_S$. For loading control, skeletal muscle α-actin was used. The data showed that the absence of GATA6 is linked to altered expression of α-actin (Figure 1). Indicating that smooth muscle protein expression is not normal in absence of GATA6. Thus, samples 3h and 3z from stillborn babies have abnormal skeletal muscle (including heart) protein expression that may explain the cause of their death.

3.2. Overexpression of GATA6

In this study, we discovered the overexpression of GATA6 in some samples. This overexpression can prevent clear visualization of the two isoforms of GATA6 showing a band tab difficult to distinguish both isoforms (samples 1L, 1N, 1M in Figure 3). Interestingly, it turns out that the overexpression of the type C isoform alone can lead one to believe that the overexpression of the two isoforms is what was revealed by the samples which gave a strip tab by western blot (data not shown).

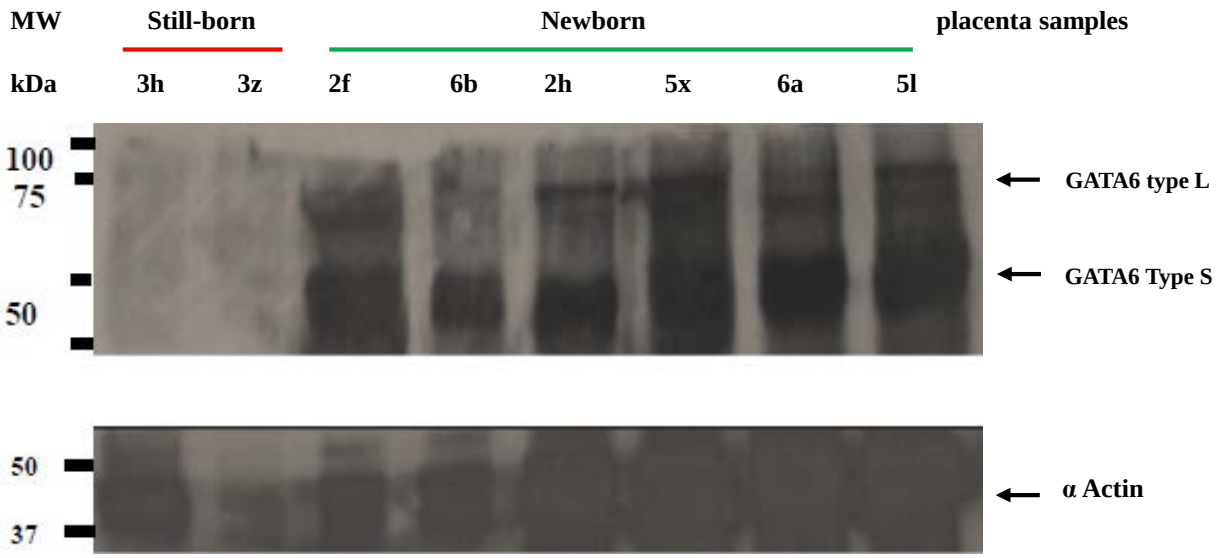

Figure 1. Western blot with 10% polyacrylamide gel showing GATA6 protein expression pattern in the placenta samples. The two isoforms of GATA6 are observed in sample 5z, 6A, 5x, 2H, and 2F (the Long type and the Short type). Sample 6B shows the loss of GATA6 type L, 3H and 3Z show undetectable expression of GATA6 by western blot. As loading control α-actin band was used

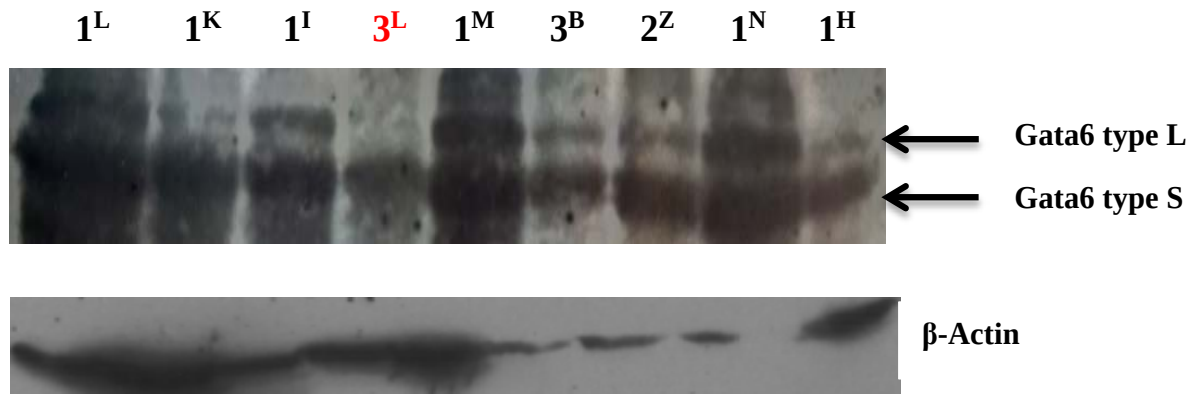

Figure 2. Western blot with 10% polyacrylamide gel showing newborn (stillborn) placenta sample without $GATA6_L$ (in red). Furthermore, samples 2z, 3B and 1I have normal expression of $GATA6_L$ and $GATA6_S$. Sample 1H and 1 K have low expression of GATA6l, while samples 1L, 1M, and 1N have an overexpression in GATA6. β-Actin is used as a protein loading control

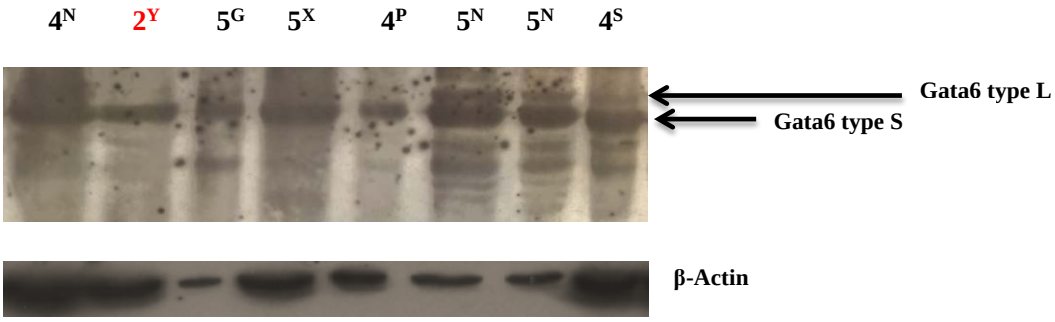

Figure 3. Western blot with 12% polyacrylamide gel showing a variation of GATA6 profiles in newborns. Sample 4N has both L and S type of GATA6. Sample 5N and 4S have additional bands at the bottom, indicating the presence of polymorphisms. Sample 2Y (in red) with a loss $GATA6_L$, is another placenta sample of a stillborn baby

3.3. GATA6 Isoforms

In this study, we observed other shorter isoforms of GATA6 suggesting the presence of mutations that may lead to the expression of different sizes of GATA6 proteins (polymorphisms of GATA6) shown by the western blot with 12% polyacrylamide gel (Figure 3). Indeed, mutations on the GATA6 gene may generate variant isoforms (Figure 3). Sequencing will determine what mutations generate the GATA6 variants. Furthermore, the polymorphisms of GATA6 in human placenta is associated to newborn deaths.

Histogram showing the frequency of GATA6-L isoform expressions in maternal placenta and the relation to newborn health status is presented in Figure 4. The absence of expression is linked to stillborn (in red); the low expression is associated to cardiopathies or metabolic syndromes (in blue), the normal expression is not linked to sick children (in green) while the overexpression is associated to cardiac hypertrophy (in yellow). However, more studies are needed to better understand the implication of GATA6 polymorphisms in newborn development.

3.4. Consequences Linked to the Deficiency of Type L Isoform of GATA6 in Placenta Samples

Several studies have reported that the early deficit of GATA6 during animal embryonic development leads to embryonic lethality following failure in forehead, heart and gut formation. But no study has successfully established the loss of GATA-6 type L by immuno-blot in a human embryo or maternal placenta. In this study we analyzed the expression of GATA6 in 6 placental protein lysates from different stillborn babies. Curiously, 4/6 of them displayed the loss of type L isoform of GATA6 (Figure 2 and Figure 3). In two of them (2/6) congenital encephalopathy was observed. Overall placenta lysate of all living newborns expressed the type S isoform of GATA6 in all 80 samples while variable expression frequency was observed for the type L isoform of GATA6 (Figure 4). Normal expression of Type L isoform of GATA6 was observed in 63.8% of the samples; overexpression was observed in 7.5% of the samples; low expression was in 20% of them and totally lost in 8.7% of the samples.

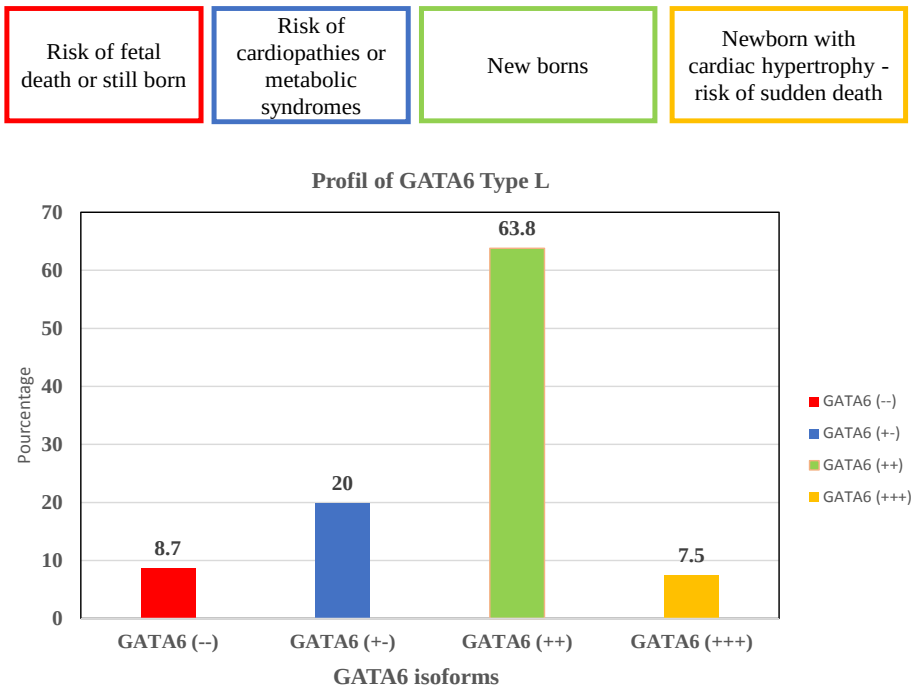

Figure 4. Histogram showing the frequency of GATA6-L isoform expressions in maternal placenta and the relation to newborn health status. The absence of expression is linked to stillborn (red); the low expression is associated to cardiopathies or metabolic syndromes (blue), the normal expression is not linked to sick children (green) while the overexpression is associated to cardiac hypertrophy

4. Discussion

Up to today, no study has illustrated the polymorphism of GATA6 associated with congenital heart disease and metabolic syndromes in human newborns using the western blot method. Animal experiments have revealed critical roles for GATA6 in myocardial development and cardiac morphogenesis, highlighting the potential involvement of GATA6 defects in the pathogenesis of congenital heart disease [19,20]. Also, it has been shown that the overexpression of GATA6 is sufficient to induce cardiac hypertrophy in the neonatal rat [21,22]. Many studies have not focused on the overexpression of GATA6 mainly that of type L. Some study reported that, a monitored overexpression of GATA6 in the murine neonatal myocytes induced cardiac hypertrophy with aging and predisposition to greater hypertrophy [21].

Herein, we discovered western blot technique can be used to assess the overexpression of GATA6 in the placenta of newborns. These results suggest that spontaneous or late cardiac hypertrophy could be a programming of fetal genes. Indeed, the overexpression of GATA6 in this study indicates the disruption of cardiac homeostasis in utero and could predispose these newborns to cardiac hypertrophy even before adulthood. This study demonstrates that the overexpression of GATA6 type L observed in few samples could be linked to hypertrophic cardiomyopathies independently of other genetic factors and could lead to sudden death [23]. These hypertrophic cardiomyopathies are diseases due to biochemical and epigenetic mechanisms [23]. Nevertheless, the assessment of embryonic biomarkers such as GATA6 expression, would be a great innovation to uncover early hypertrophic cardiomyopathies in newborns.

Study has reported GATA6 mutation in a patient diagnosed at 1 month old with a ventricular septal defect, a persistent arterial duct, a congenital diaphragmatic hernia at birth, permanent neonatal diabetes mellitus and exocrine pancreatic impairment due to pancreatic agenesis [24]. Furthermore, GATA6 deficiency is not only the cause of pancreatic agenesis and congenital heart defects but can also affect other organs derived from the endoderm [24]. As, GATA6 is necessary for the endodermal and mesodermal cell lineage determinations [25,26,27]. *In vitro* knockdown of GATA4 or GATA6 in murine embryonic stem cell models impact liver and heart gene expressions [7,25].

For the first time, our study reported the loss of placental GATA6 associated to four stillborns in human. This finding rose the assumption that the absence of heart lineage specification transcription factor GATA6 may have role in their death. This confirms that GATA6 deficit impacts the survival of embryos ranging from a simple heart defect to premature death. Newborns with GATA6 anomalies we have observed in our study, may be programmed for asymptomatic congenital heart disease or metabolic syndromes that put them at risk of sudden cardiac arrest or diabetes later in life.

It is obvious that the deficit of GATA6 discovered in placenta linking the mother and the newborn is an indicator for possible congenital heart disease and other embryonic organs failure including the pancreas, the diaphragm and pericardium [13,14,15]. All of which causing metabolic syndromes associated with heart disease, abdominal obesity, hyper-cholesterolemia, dyslipidemia, high blood pressure, neonatal diabetes as well as malformations such as pancreatic agenesis, diaphragmatic hernia [13,14,15]. By seeking the origin of metabolic syndromes at the molecular level, more particularly that of diabetes (types I and II), many articles and reviews have implicated GATA6 as the main molecular player [28]. As matter of fact, GATA6 controls the biosynthesis and the secretion of insulin in adult beta cells [28]. In addition, the expression of the transcription factor HNF4α involved in the differentiation of hepatocytes and their function in the metabolism of lipids and glucose requires the expression of GATA6 [25,29]. In reality, GATA6 is located upstream and regulates the expression of HNF4α directly or indirectly and any abnormalities of GATA6 will inevitably lead to an abnormal expression of HNF4α which will subsequently lead to hepatic disorders such as diabetes and metabolic dyslipidemia [25,29,30,31]. Amazingly, GATA6 deficiency in two newborns did not lead directly to their death and suggested that it is not at gene level but rather, the low level of protein expression was not detected by the immunoblotting. Nevertheless, these data has drawn our attention to the fact that low GATA6 will be linked to low HNF4α and can thus predispose these newborns to diabetes even before adulthood independently of family determining factors [25,29,30,31].

Ultimately, GATA6 isoforms were also observed in two newborns placenta samples suggesting the presence of mutations on the gene with synthesis of shorter isoforms linked to polymorphism. Interestingly, no previous published data on GATA6, has illustrated polymorphic mutations on human GATA6 gene signaled by protein isoforms observed by Western blot. However, this polymorphism can lead to a gain or loss in function of the GATA6 protein. Indeed, functional gain mutations of GATA6 was observed in a family with early atrial fibrillation and atrioventricular septal defects, as well as in a family with early and sporadic atrial fibrillation as determined by complete sequencing of the exome [32]. The mean age of the onset of atrial fibrillation in these families was 47.1 years [32]. Simple Nucleotide Polymorphism (SNP) type was found through genetic and functional analysis in the promoter of the GATA6 gene in a large group of patients with a ventricular septal defect, a form of congenital heart disease [33]. Therefore, the isoforms of GATA6 found in our study may reflect mutations in the GATA6 gene and predispose these newborns to congenital heart disease and other congenital anomalies later in life. This observation is strengthened by other study which demonstrated that a mutant GATA6 protein was approximately 15 kDa smaller than the wild type (52-55 kDa) causing in patients a congenital heart disease characteristic called persistent arterial truncus [34]. Thus, the protein bands found in this study that are not in the same molecular size positions of wildtype GATA6, would be mutant isoforms of GATA6 protein due inexorably to a mutation in a wild type GATA6 gene. Functional in vitro analyzes have shown that each mutation on GATA6 gene can lead to a functional disturbance of the GATA6 protein and modulate the regulation of its target genes during embryogenesis [25,34,35].

It is important to emphasize the fact that congenital heart diseases and metabolic abnormalities are not pathologies that appear suddenly throughout adulthood because of unfavorable lifestyle of adults but rather a biochemical or epigenetic programming of the embryos even before their births or during lifespan. As matter of fact, epigenetic silencing of GATA6 in ovarian epithelial cell of adult women can be corrected with histone deacetylase drugs before conception. Therefore, in consideration to GATA6 function as transcription factor in the regulation of cardiac genes in the early developing human embryo, it should be thoroughly investigated in women prior to conception (periconceptual period). GATA6 status should be investigated early in pregnant women to correct it deficient status with proper nutritional supplements or specific drugs to limit stillborn or sudden death among newborns.

The findings in our study will contribute undoubtedly to establish a new paradigm of placenta screening for biomarkers to predict the risk of heart defect and metabolic syndromes early in life to reduce morbidity and mortality in our population.

5. Conclusion

In this study, the value of GATA6 as a potential prenatal, neonatal or postnatal biomarker for congenital heart disease and metabolic syndromes is revealed. GATA6 is an interesting biomarker for the pre-screening and the diagnosis of pregnant women who are at risk of losing the embryo before the end of pregnancy or at birth, due to hearth defect. Genetic deletion of GATA6 is embryonic lethal. However, molecular deficiency of GATA6 due to poor nutritional status or environmental influences, could be restored with proper nutrition. It is urgent to redirect nutritional programs of pregnant women to save newborn life and extend survival for those with multiple isoforms of GATA6 suggesting the presence of polymorphism linked pathologies.

Ethical Statements

This study obtained the approval of the Research Ethics Committee of the Benin Institute of Applied Biomedical Sciences (CER-ISBA) before sample collection.

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Abbreviation

APS: Ammonium persulfate
DOHD: Developmental Origins of Health and Disease
HNF4: Hepatic Nuclear Factor
GATA6: GATA binding protein 6
GATA4: GATA binding protein 4
PBS: Phosphate Buffered Saline
RNA: Ribonucleic acid
TBS : Tris Buffered Saline
TBS : Tris Buffered Saline Tween-twenty
RA: Retinoic Acid

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