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Comparison of the Folate and Homocysteine Levels with A80G - RFC1 Gene Polymorphism between the Sample of Iraqi Children with and without Down Syndrome

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Abstract

Many international studies indicated that the polymorphisms of some genes disturbed the folate homocysteine (Hcy) metabolism and increased the vulnerability to Down syndrome (DS). We aimed to measure the serum levels of folate and Hcy in DS children and compare the levels with age and sex-matched apparently normal healthy children. We also aimed to study the A80G polymorphism of the gene reduced folate carrier (RFC1) in the DS children as a risk factor. Forty children with DS (24 were boys, and 16 were girls) with the age range between 5-13 years, and 26 normal healthy children (16 boys and ten girls) were included in this study. The results show that the highest genotype in the control group was AG (53.85%) followed by AA and GG (30.77% and 15.38%) respectively. The genotype percentages in children with DS were (50, 35,and 15) for AG,AA, and GG respectively. There was no statistically significant Down syndrome risk RAC-1A80G polymorphic genotype and DS in the Iraqi children sample (A/A Vs. G/G, OR= 1.13,95%C =0.48-2.68) (A/A Vs. A/G, OR= 1.2, 95%C = 0.66-2.26)(G/G vs. A/G, OR=1.08, 95%C = 0.48-2.43) respectively. Significant decrease in the concentrations of Hcy and folate in the serum of DS children 5.54 ± 0.94 and 6.99 ± 1.16, then in the control group 7.14 ± 1.46, 7.86 ± 1.78, respectively. We did not detect a significant difference between male and female DS subjects. There was no correlation between the Hcy concentration and folate level of the DS group. The results showed that the frequency of RFC1 alleles and A80G genotypes (GG Vs AA, AAVs AG, GG Vs AG) had no risk with down syndrome in a sample of Iraqi children. Thus, we need further studies with a large sample of children in comparison with mothers of DS birth.

Keywords: RFC1 80A-G Polymorphism, Down Syndrome, Serum Folate, Homocysteine Levels, Iraqi patients

1. Introduction

Trisomy 21, known as Down Syndrome (DS), is the most common human chromosomal disorders in born children (1 in 800–1000) (Capone, 2004; Sadiq *et al*., 2014). It has been assumed that the interactive role of various genetic and environmental factors is associated with DS (Sadiq et al.,2019). Folic acid (globolutamic acid), as a dietary factor, plays a vital function in the progress of distribution of genetic materials during cell division due to its role in cellular methylation reactions. Folic acid is implicated in epigenetic regulation and other processes in the synthesis and repair of DNA (Argellati *et al*., 2006; Sadiq *et al*.,2019). The loss of chromosomal balance and the disturbed gene expression of extra chromosome 21 are products of mitotic error in the embryo development. The gene expression of extra chromosome 21 is a result of the failure of normal chromosomal segregation during maternal meiosis. Alternatively, oxidative stress caused by DNA damage plays a crucial role in clinical manifestation of DS (Biselli et al., 2008; Zitnanova et al., 2006), due to over-expression of antioxidant enzyme present in chromosome 21-associated with many other clinical traits including mental retardation and congenital heart disease (Carratelli *et al*., 2005).

Chromosome 21 contains 225 genes, some of which are believed to be located in the critical area of Down syndrome (DSCR), implicated in the pathogenesis of DS. However, the functions of most of the encoded proteins are still unknown. DSCR contains genes coding for Folate is significantly implicated in cellular methylation reactions (Fenech, 2005). Folic acid acts primarily as a single carbon unit donor involved in many essential body processes, including DNA and RNA synthesis and repair. Its metabolism is associated with the primary methyl group donor for methylation reactions of DNA, lipid, and proteins. Proteins that carry folic acid are also crucial in sustaining DNA methylation because they determine the level of folate present in the cells (Yates, Z., and Lucock , 2005; Chango *et al*.,2000). Recent evidence indicates that up to ninety percent of children with psychological and DS are born from young mothers, and have other risk factors than maternal age (Hobbs *et al*., 2000 &2002).

Some studies have suggested a relationship is found in the presence of DS and some mutations or polymorphism in genes of mother implicated in the mechanism of the

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metabolism during folate acid transport and synthesis (Scala *et al*., 2006; Santos-Reboucas *et al* ., 2008).

Homocysteine (Hcy) is a sulphuric amino acid produced via folate metabolism and could undergo reconstitution to methionine or conversion to cysteine in the transsulfuration pathway. Hcy concentration is affected by modifiable and non -modifiable factors such as vitamin status, sex, and genetic factors (Yelina *et al*., 2012).

Among the genes involved in the folic acid metabolism, reduced folate carrier 1 (RFC1) also known as SLC19A1 is by implication involved in neural tube defects, Down syndrome, and leukemia. RFC1 is found in the intestinal mucosa membrane. According to Nguyen et al. (1997), RFC1 is involved in folic acid absorption by conveying 5-methyltetrahydrofolate into the cells. The A80G variant of the RFC1 gene could also be linked to the changes in products got in this metabolic pathway (Coppedè *et al*., 2006; Coppedè *et al*., 2013).

Different research works have shown the pathogenicity of folate or the function of folate in preventing neural tube defects. Abnormal Folate and homocysteine metabolism lead to DNA strand breaks, chromosomal instability, impede DNA repair capability, DNA hypomethylation, and abnormal gene expression (Moustafa *et al*., 2016; Kazemi *et al*., 2016). Consequently, the current work aims to evaluate the serum levels of folate and homocysteine in children with DS and compare them with normal controls. For the first time, the RFC1 gene A80 G polymorphism was evaluated in Iraqi children with DS.

Subjects, Materials, and Methods

The study included a patient group of 40 children with DS from Baghdad Teaching Hospital –Medical City-Ministry of Health, and from Al-Safa center in Zayoonah city, Baghdad, Iraq. This study was performed from April 2019 to the end of September 2020.

The control group included 26 (16 boys and ten girls) Iraqi pupils from 5 and ^{sixth} class Zamzam primary schools in Baghdad city, with the age range between 10 to 14 years.

All pupils who were involved in this study were intervi ewed to allow for case-control design by comparing serum folate and homocysteine levels as well as RFC1.

1.1. Samples collection

We collected five milliliters of the venous blood sample from each child in the two study groups carried out. Two ml- serum was used for biochemical analysis and two ml of the blood sample was transferred to EDTA tubes for molecular analysis to detect RFC1-A80G Polymorphism.

1.2. RFC-1 Gene Polymorphism

The genomic DNA was isolated from 66 blood samples collected in EDTA anticoagulant tubes from the two study groups, according to the protocol of the Relia Prep TM Blood gDNAMiniprep System Kit (Promega, USA). Polymorphism analyses by using PCR-RFLP, the amplification of primers were as reported by Neagos et al. (2010): forward of primer: 5'-AG TGT CAC CTT CGT CCC-3' and the sequence for reverse 5'-TCC CGC GTG AAG TTC TTG-3'.

The polymerase chain reaction for RFC - 1 gene A80G polymorphism was performed in a total volume of 25 µl, with PCR conditions as follow: one cycle of 94°C for 2 minutes as a preliminary DNA denaturation step, followed by 44 cycles of 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 55 seconds, which is followed by 1 cycle at 72°C for 7 minutes for a final extension. Five µl from each sample PCR product were electrophoresed to ensure the positive reaction of amplification. The PCR products were digested with *Cfo I* (Sigma, USA) with 0.5 µl restriction enzyme, which was added to 20 µl of PCR products for 3 hr. The incubation period generated three parts of 125, 68, and 37 bp, with the G allele, while the A allele generated two parts of 162 and 68 bp. Amplicons were electrophoresed, and visualization was done by gel 2 % agarose which contains the ethidium stain bromide; after that, we reached the results from comparison to the ladder of 100- base pair.

Serum Hcy and folate levels were measured according to the principle of immunoassay kits using Immulite analyzer –Siemens-Germany in teaching laboratory-hormonal and biochemical assay for the ministry of the health-medical city.

1.3. Statistical Analysis:

The Statistical Analysis System- SAS (2012) method was adopted for the detection of the impact of various indicators in study parameters. T-test was utilized for a substantial comparison between means. The estimate of correlation coefficient-r in this study and the chi-square test were utilized in comparing allele and genotype frequenters between the two study groups. Odds ratio with 95% confidence interval in the DS group. $P<0.05$ was regarded as statistically significant (SAS. Version 9, 2012).

2. Results

Table 1 illustrates the allele numbers and RFC1genotype frequencies for control and Down syndrome children by using Hardy-Weinberg equilibrium. The percentages of genotypes involved in this polymorphism for healthy normal children were the A-G (53.85%), A/A (30.77%), 15.38% ,and G/G genotypes respectively.

The children with DS group have a higher percentage of the genotype A/G (50%) followed by A/A (35%), then G/G genotypes (15%), without any statically significant differences about the polymorphism A–G in the RFC1 gene when compared Down syndrome to healthy control children.

Table1. RFC1 rs 1051266, A/G genotypes and allele frequencies among study groups

Group	Genotype (%)			Allele frequency		Persons chi-square X^2(df=2)	P
	AA	GG	AG				
	N (%)	N (%)	N (%)	A	G		
Control N=26	8(30.77)	4(15.38)	14(53.85)	0.58	0.42	0.15	0.93
Patients N=40	14(35)	6(15)	20(50)	0.6	0.4		

From the odds ratios calculation (table 2), the study subjects who carried the A/A or A/G genotypes of RFC1 gene have a non-significant effect (p>0.05) Down syndrome risk (OR= 1.2, 95% CI=0.66-2.26), (OR= 1.08, 95% CI= 0.48-2.43) in comparison to individuals who carried A/G genotype.

The results showed that there was non-significant Down syndrome risk (OR= 1.13, 95% CI=0.48-2.68) when compared the subjects who carried the A/A genotype of RFC1 gene versus individuals who carried the G/G genotype.

Table 2. RFC1 A and G allele polymorphism in Down syndrome group

	Genotypes	**OR**	**95%CI**	**P**
Down syndrome	A/A Vs. G/G	1.13	0.48-2.68	0.78
	A/A Vs. A/G	1.2	0.66-2.26	0.53
	G/G Vs. A/G	1.08	0.48-2.43	0.85

The mean Homocysteine concentration in DS children showed a significant decrease (5.54 ± 0.94), compared to the control group (7.14 ± 1.46) (P = 0.01). The serum levels of folate were significantly lower in the DS group (6.99 ± 1.16) when compared to the control group (7.86 ± 1.78) as presented in table 3. However, there were no statistically significant differences between the concentration of Hcy and folate with gender in DS or normal healthy children (Table 4). There was no significant correlation between the Hcy concentration and folate level of the DS group and controls, as shown in table 5.

Table 3. Comparison between DS and control in Homocysteine and Folate

Group	Mean ± SD	
	Homocysteine	Folate
DS	5.54 ± 0.94	6.99 ± 1.16
Control	7.14 ± 1.46	7.86 ± 1.78
T-Test	0.591 **	0.725 *
P-value	0.0001	0.0206

* (P<0.05), ** (P<0.01)

Table 4. Comparison between DS and control in Homocysteine and Folate

Group	Sex	Mean ± SD	
		Homocysteine	Folate
DS	Male	5.83 ± 0.82	7.11 ± 1.34
	Female	5.10 ± 0.95	6.82 ± 0.85
	T-Test	0.575 NS	0.768 NS
Control	Male	7.34 ± 1.52	7.90 ± 1.88
	Female	6.84 ± 1.40	7.79 ± 1.72
	T-Test	1.228 NS	1.515 NS

** (P<0.01), NS: Non-Significant

Table 5. The correlation coefficient between Homocysteine and Folate in patients and control

Group	Parameters	Correlation coefficient-r	Level of sig.
DS	Homocyst and Folate	0.19	NS
Control	Homocyst and Folate	0.13	NS

NS: Non-Significant

3. Discussion

Folic acid is a coenzyme in the folate metabolism that works by transferring the active form of folic acid to cells, which can affect the activity of enzymes that take a critical function as a risk factor for homocysteine level and many other diseases such as defects in neural senses and cardiovascular diseases (Zampieria et al., 2012).

The gene included in the metabolism of folic acid and folate is RFC1, also named SLC19A1; the purpose of our work is to compare the presence of RFC1 rs1051266, A80G polymorphism in DS children and healthy (Locke et al., 2010). In the present study, as appearing by chi- sequre test, it was found the allele A was more frequent than allele G in both DS and control children groups. No significant difference was detected in A80G polymorphism between the two groups.

Also, Neagos et al. (2010) indicate that the G allele may alter and increase the risk and probability to born child with DS from mothers over 34 in southern Italy. However, Saghazadeh et al. (2017) considered the presence of the G allele as a risk factor for nsCLP in Iranian infants but considered the result to be commendable due to other conditions such as gene-environment interaction and amount of folic acid that play a role in the etiology. Similarly, in the Indian population, Lakkakula et al. (2015) demonstrated the association of allele G with nsCLP, and they consider the allele G to be associated with the Asian population.

Additionally, it was found that the AA is the most common genotype in DS children (35%) than the control group (30%). However, this was not a statistically significant difference. In recent years, due to the increased

occurrence of children with DS from mothers at an early age, it indicates that other factors are acting, as DS mainly occurs at older age, leading to the study of physiological conditions and mechanisms that are best to grow DS fetuses. The studies on cell cultures demonstrated the folate deficiency is eligible to stimulate chromosome 21 aneuploidy, and the results assumed DS has many causes linked to genetic and acquired factors such as epigenetic or environmental and random origin. Therefore, it is difficult to establish and identify the effect of the first contribution to each of these factors (Varga *et al*., 2006).

The variation in the allele and genotype distribution maybe due to the differences in the sample of this study and genetic background differences of children adding to the impact of complicated environmental factors.

In our study, mean folate level in DS children was substantially lower than in the control group; this result agrees with study by Fillon-Emery et al. (2004) and Varga et al. (2008). However, this result disagrees with the results of Gueant et al. (2005) who did not observe the significant differences between DS and healthy individuals in the folate level while the level in this study let down than the folate concentration in the study of Kumar and his group (2014) in Indian children with DS, who proved active folate deficiency despite normal plasma amount of folate.

The folic acid level in this study was found to be below the biological range value in healthy children and DS children, and this can be explained by the fact that all cases and the control group were children with poor conditions, malnutrition, and wars with shelling Essential sources of dietary folates are fruits, beans, cereals, green vegetables, and calf liver which is referred to as single carbon metabolism, to produce the main intracellular methylating like the S-adenosylmethionine (SAM). At the same time, DNA is also producing the precursor of the RNA (Pogribna *et al*., 2001). Folate is considered one of the molecules having the hydrophilic characters which were not able to pass the membrane of biological object through promulgation alone unless by using various transport system to get in the cells.

In this study, the mean of serum Hcy concentration was significantly decreased ($p<0.01$) among children from control, and the current result of lowering the Hcy concentration of children with DS agrees with the study of Pogribna et al. (2001) and Meguid et al. (2010).

Similarly, Nandha Kumar et al. (2014) observed hyperhomocysteinemia in DS. They explained that it could be as a result of the elevation in the transsulfuration for the Hcy pathway, which originates from the increase in the expression of cystathionine B synthase on chromosome number 21 which leads to deficiency and decrease in the functional folate Also explained that decreasing and increasing folate concentration and Hcy with the advancement in the children's age, which was described by the increased requirement for folate in the early stage of the growth and development of infants. The results of the present study about gender variations in the concentration of folate and Hcy between boys and girls of DS children or in normal healthy were statistically not significant, and this may be caused by that in our study all samples of children were at the stages of growth and development, in Iraq

Gueant et al. (2005) and Laraqui et al. (2006) revealed that a high Hcy level may be harmful and is particularly linked to the elevated risk for heart diseases and psychiatric with neurodegenerative disorders. According to the Hobbs et al. (2000 and 2002) study, the presence of genetic variants in genes implicated in the folate metabolism among individuals with DS may lead to a survival advantage. Hcy is responsible for a critical role in folate-dependent DNA synthesis. Hcy plays an essential part in the reactions of cellular methylation that are essential for the growth of the fetus and the interaction of fetal and maternal genotypes.

The preferable recognition of folate transporter was expressed reduced RFC-1 which takes part in the regulation and function of the cofactor of the folate involved in the blood (Stanger, 2002). Also, guide work of RFC-1 functions of specialized tissue like assimilation of the luminal layer of the intestine and the uptake for folate through the process as barriers between blood and the brain organ transplacental transmission of folate and travel through some tubules and membranes in the kidney. Decreasing of folate in cells outcome from disequilibrium in the DNA methylation process, chromosome breakage, point mutations, aneuploidy, and chromosome recombination defect while related to various diseases of humans such as cardiovascular diseases and congenital diseases, neuropsychiatric disorders, and cancer (Santos-Reboucas *et al*., 2008).

4. Conclusion

The results of our work revealed a substantial decline in the Hcy and folate levels in DS children when compared to the control group, but not significant with the gender. There was no correlation between the Hcy concentration and folate level of the DS group.

The results showed that the frequencies of RFC-1 alleles and A80G genotypes (GG Vs AA, AA Vs AG, GG Vs AG) had no risk with Down syndrome in a sample of Iraqi children. Thus, we need further studies with a large sample of children in comparison with mothers of DS birth.

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