THE IMPACT OF FOLLICLE STIMULATING HORMONE RECEPTOR GENE POLYMORPHISM ON PROGRESSION OF PRIMARY AMENORRHEA IN A GROUP OF IRAQI FEMALES: A CASE CONTROL STUDY

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Abstract

This research designed to investigate the impact of single nucleotide polymorphism (rs6166) of the Follicle Stimulating Hormone Receptor (FSHR) gene in a group of females diagnosed with primary amenorrhea. Blood samples were collected from healthy control group consisted of 40 females between the ages of 12-35 who appeared to be in good health, while 62 patients who visited the educational laboratories at the City of Medicine in Baghdad from October 2018 to February 2019. In the meantime, molecular analyses were performed on all blood samples. SNP (rs6166) determinations were performed using direct sequencing. Calculations of allele frequency for exon10 rs6166 single nucleotide polymorphism showed that G and A alleles was not associated with the disease with *P* value (0.306) for both alleles and also showed no significant association for the AA , AG and GG genotypes with the disease with *P* (0.650), (0.675) and(0.261) respectively. Comparing between genotype variations and hormones level in serum in control group showed a significant association of AG genotype with FSH level with *P* value(0.031), while in patient group the comparison showed significant association of AA and AG genotypes with LH level. The comparison of age categories with genotype variations for both control and patient groups showed no significances with *P* >0.05. All these results confirmed that rs6166 polymorphism could not considered as a molecular marker for primary amenorrhea in Iraqi females.
Keywords: Primary amenorrhea, Follicle Stimulating Hormone Receptor (FSHR) gene, SNP, Iraqi women patient.

Introduction

Primary amenorrhea(PA) means the lack of secondary sexual characteristics' growth or development by the age of 14, or the lack of secondary sexual characteristics' growth or development by the age of 16, despite the presence of normal growth and development, including secondary sexual characteristics (Samal and Habeebullah, 2017). According to world health organization(WHO), 25% of female infertility caused by amenorrhea that affects women in the reproductive age. Primary amenorrhea and menstrual irregularities affected 9.68 percent and 17.78 percent of women in Iraq in 2017, respectively, according to statistics from the ministry of health for the years (Mu *et al.*, 2019).

Gonadotropin-releasing hormone (GnRH), which is released from the hypothalamus during a typical female menstrual cycle, acts on the pituitary to release follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These two hormones from the pituitary then act on the ovaries to produce estrogen and progesterone, which then work on the uterus to carry out the follicular and secretory phase of the Amenorrhea can result from any malfunction in this healthy physiology at any level (Reed BG, 2018).

A woman of reproductive age who is not menstruating may have hormonal, physiological, or anatomical problems that disrupt her body's regular functions. The hypothalamus, pituitary, ovaries, and uterus work together to balance hormones and provide feedback to the normal physiological mechanism (Nawaz G, 2019) (Van der Wijden and Manion, 2015).

The rhodopsin-like receptor subfamily's 76 kDa follicle stimulating hormone receptor (FSHR) has 695 amino acids and is a G protein-coupled receptor. The FSHR gene, which has more than 190K bases and 10 exons and 9 introns, is found on chromosome 2p21. Exons 1 through 9 encode for the extracellular domain that is tasked with ligand binding, while the massive exon 10 encodes for the seven transmembrane spanning domains, the intracellular C-terminal tail, and a portion of the "hinge region." In granulosa and Sertoli cells,

where it is mostly expressed, FSHR mediates steroid production and gametogenesis (Hammond *et al.*, 2014).

Multiple intracellular signaling pathways are simultaneously activated after ligand interaction causes a conformational change in the receptor. Asn680Ser (c.2039; rs6166) The hinge region protein receptor undergoes an amino acid change as a result of an SNP in the exon 10 of the FSHR gene. (Ulloa-aguirre *et al.*, 2018).

Materials and Methods

To study molecular parameters, this experimental work was done in the educational laboratories of the City of Medicine in Baghdad from October 2018 to February 2019. The healthy control group consisted of 40 females between the ages of 12-35 who appeared to be in good health, while 62 patients who visited the educational laboratories at the City of Medicine in Baghdad were found to have primary amenorrhea. All of the patients had primary amenorrhea symptoms. In the meantime, molecular analyses were performed on all blood samples. According to the manufacturer's instructions, the Wizard genomic DNA purification kit (Promega, USA) was used to extract the genomic DNA from blood. Primers were created using NCBI and provided by the Macrogen firm in lyophilized form. They were then diluted in sterile distilled water to a final concentration of 100pmol/ul for the detection of rs6166 in exon 10 of the FSHR gene (Zaker *et al.*, 2010). Primers used for detection of rs6166 SNP are listed in table 1

Table 1: the sequences designed primer for rs6166 SNP.

Name	Sequences	GC %
R	ˈ5-GTGGCTGCTATATCCACATC-ˈ3	50%
F	ˈ5-CTATCCACACTGACGCATTAC-ˈ3	50%

The samples underwent a standard polymerase chain reaction (PCR).The amplification of the FSHR gene was done according to Kiyoi *et al.* (1997). The annealing temperature was determined by optimization to the primer as shown in Table(2).

Table(2): The PCR Reaction Program.

Program steps	Temperature ^{0}C	Time	No. of cycles
Initial denaturation	95	5min	1
denaturation	95	30s	
annealing	55	45s	
extension	72	1min	35
final extension	72	7min	1

Agarose gel electrophoresis with 1% concentration was used to analyze the product of PCR, and run at 90 volts for 1.5 hours. The PCR products of the analyzed *FSHR* regions and primers were sent by Macrogen Corporation – Korea for sequencing, These samples' sequences were compared to the data for the standard FSHR gene in the gene library of the National Center for Biotechnology Information (NCBI).

Results

The polymerase chain reaction was done under optimal amplification conditions by using a set of specifically designed primers for rs6166 SNP. Results illustrated in figure (1) showed the amplified products were appeared as a sharp and clear band, with a molecular size of 988bp of Exon10.

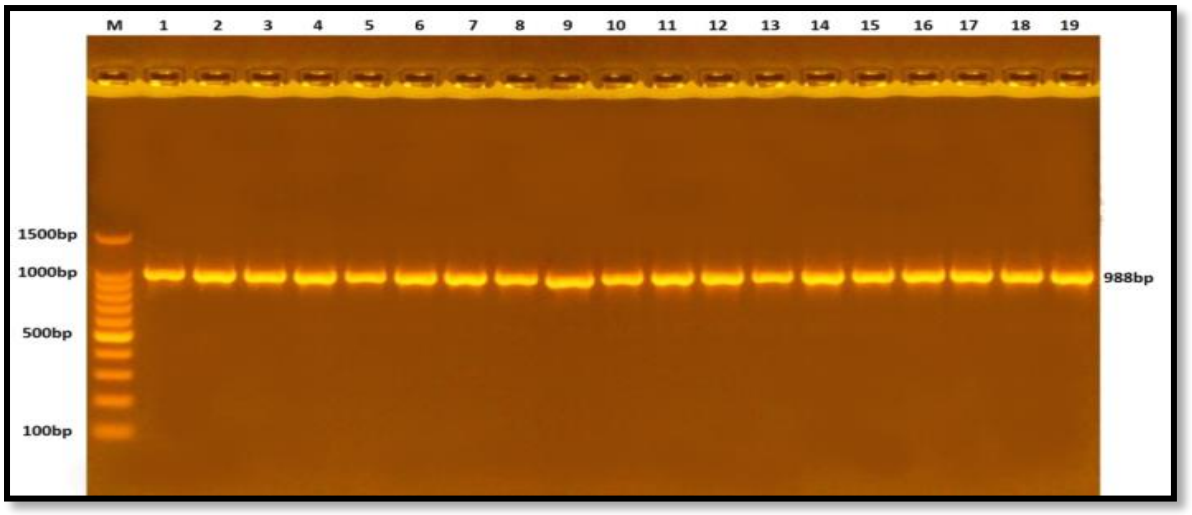

Figure 1: Electrophoresis on agarose gel (1%).

results illustrated in figure(2) showed the nucleotide sequence of the specific region including rs6166 in PA patients and healthy controls.

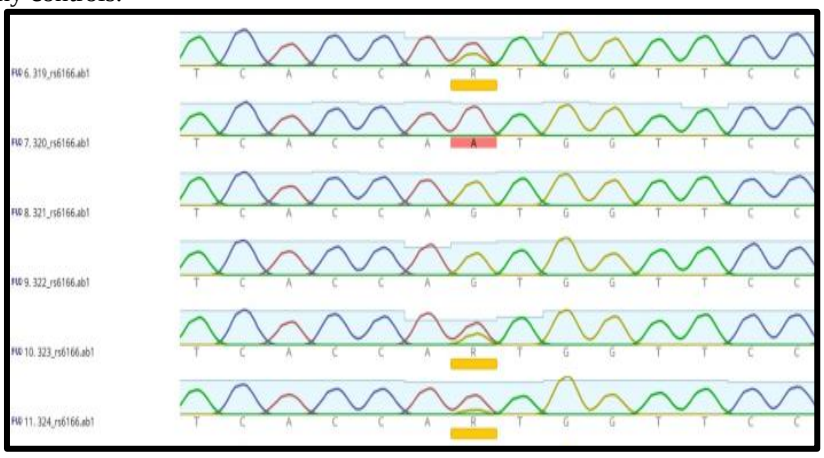

Figure 2: Nucleotide sequence of the specific region of Exon10 for PA cases.

The rs6166 SNP in *FSHR* was genotyped in a randomly selected group of controls and PA patients.

On chromosome 2, the FSHR gene's SNP (A>G rs6166) was shown to have three genotypes (AA, AG, and GG) and two alleles (A and G). The genotypes were compatible with the Hardy-Weinberg equilibrium (HWE) in the healthy and PA groups, and there were no significant discrepancies ($p > 0.05$) between the actual and anticipated genotype frequencies table (3A).

Table 3A: Comparison of primary amenorrhea patients with the control group for the number and percentage frequencies (observed and predicted) of the A>G genotypes of the FSHR gene SNP (rs6166) and their Hardy-Weinberg equilibrium (HWE).

Genotype	Patient(no=56)				Control(no=40)			
	Observed		Expected		Observed		Expected	
	No.	(%)	No.	(%)	No.	(%)	No.	(%)
AA	15	33.93	16.1	28.75	13	32.5	12.1	30.25
AG	22	39.29	27.9	49.82	18	45	19.8	49.5
GG	19	26.79	12.1	21.61	9	22.5	8.1	20.25
HWE-Analysis	*p*=0.116 Non-Significant				*p*=0.565 Non-Significant			

There was no discernible difference in the genotypes and allele frequencies of the FSHR gene in the (PA) and control groups. in the homozygous genotype frequency of A, G allele ,and in the heterozygous genotype frequency of A and G allele., Despite the fact that patients' G allele frequencies were lower (54.0 vs.

45.0 percent) and their A allele frequencies were higher (27.5 vs. 41.7 percent) (Table 3B). In the rs6166 polymorphism, the odds ratios for the genotypes AG, AA, and GG were 0.79 (0.35 - 1.78), 0.76 (0.32 - 1.83), and 1.77 (0.71 - 4.42), respectively, with p=0.675, p=0.650, and p=0.261, respectively, showing that neither the heterozygous genotype AG nor the homozygous genotypes AA, GG.

Table (3B): Genotype and allele frequencies of 6166 SNP in PA and control group.

Many studies in the world showed great attention for rs6166 polymorphism and its relation to many problems in the female reproductive system and infertility, This polymorphism, which is present in the intracellular portion of the receptor, has the potential to change the intracellular portion's glycosylation state and, as a result, the downstream signaling response and receptor activity that occur when FSH attaches to its receptor(Lindgren, 2019). The change from A to G at position 2039 causes a change from Asparagine to Serine at position 680 in the intracellular domain of the receptor which in turn introduces a potential phosphorylation site. This change, though involving a change in only one base pair each, could affect the downstream mechanisms of the FSH receptor which could, in turn, affect the menstrual cycle resulting with the patient presenting with primary amenorrhea (Simoni and Casarini, 2014).

According to Thomas, *et al.* (2014) study that done on Indian female population, results showed that 42.4% of the patients with PA were found to have a heterozygous condition. Since these patients have one allele that is normal and functioning, this heterozygosis cannot be attributed as the cause for PA in them and other causes should be explored. These results also authenticated by another study Sudo, (2002) done on Japanese females suffering from primary amenorrhea, indicates that there is no association between observed genotypes and disease of interest, however, the SNP showed strong significance with secondary amenorrhea (polycystic ovary syndrome PCOS). 450 Chinese women who were infertile were studied in 2013(Yan *et al.* 2013) demonstrated a correlation between the polymorphism rs6166 and the ovarian response to FSH. When compared to genotype AA, genotype GG at position 680 showed greater levels of basal FSH and poorer response rates, also found that higher response to FSH was associated with AG genotype. Even though this polymorphism did not have any relation with PA but it could affect other diseases that influence the reproductive system of women, that leads to problems with menstrual cycle and infertility.

Endocrine profile linked statistically with genotype to determine the influence of SNP on hormone level in both control and PA groups, results showed that there is a significant impact between s. FSH level and genotype distribution with *p*=(0.03) in overall comparison, also there is strong relation of GG genotype over AG with the level of FSH in serum with p^{1}=(0.031) as displayed in table (4A). The mean±SD of AA, AG and GG was (6.32±2.00), (6.66±1.25) and (4.72±2.12) respectively, SD value of (GG) compared with FSH level was higher than other SD values, that clarify the results.

The mean± SD of genotypes compared with LH level was (6.30±2.00), (6.30±2.39) and (6.00±2.48) for AA, AG and GG genotypes respectively. There is no significant between genotypes distribution and S.LH with *p*=(0.82) as shown in table 4A.

Table(4A): Association among rs6166 *FSHR* genotypes and endocrine profile in the control group (n = 40).

	FSHR rs6166 genotype						*P* values			
Hormone	AA		AG		GG		*P*	P^1	P^2	P^3
	n=9		n=18		n=13					
FSH (mIU/ml)	tant type)		19	33.928	9	7.5	1.77 (0.71 - 4.42)		0.261	
	6.32±2.00		56	6.66±1.25	40	4.72±2.12	0.03	0.031	0.13	0.98
			100		100					
		G	60	54.0	36	45.0	1.41(0.8-2.5)		0.306	
	cy									
		A	52	46.0	44	55.0	0.71(0.40 - 1.26)		0.306	
LH (mIU/ml)	6.30±2.46		6.30±2.39		6.00±2.48		0.82	0.98	0.97	0.99

While, comparison of endocrine profile with genotypes in PA group shown in table3.4B, there are no significant differences with S.FSH level compared with genotype diversity with p=(0.9), mean± SD for AA, AG and GG was (47.06±15.27), (47.16±16.42) and (44.86±10.90) respectively. S.LH level showed highly significant differences when compared with genotypes with overall *p*=(0.005) and higher significant of mutant genotype(GG) over wild and heterozygote with *p*=(0.01), the mean± SD was (17.18±4.62), (18.26±5.41) and (25.41±7.83) for AA, AG and GG respectively.

Table (4B): Association between rs6165 and rs6166 *FSHR* genotypes and endocrine profile in the PA group (n = 56).

	FSHR rs6166 genotype			*P* values			
Hormone	AA	AG	GG	*P*	P^1	P^2	P^3
	n=19	n=22	n=15				
FSH (mIU/ml)	47.06±15.27	47.16±16.42	44.86±10.90	0.90	0.99	0.96	0.97
LH (mIU/ml)	17.18±4.622	18.26±5.41	5.41±7.83	0.005	0.01	0.01	0.99

Another outcome observed by Comparing ages of control and PA groups with genotypes resulted from direct sequencing, as illustrated in table(5A) the ages of control population divided into 3groups, ≤15year (n=4) with genotype distribution (7.69%),(16.67%) and (0%) for AA,AG and GG respectively , while 16-19years (n=7) with (30.77%), (11.11%) and (25%) for AA , AG and GG respectively and ≥20year (n=29) with(61.54%) , (72.22%) and(75%) for AA , AG and GG genotypes .

Table(5A): rs6166 *FSHR* genotypes frequencies and age categories in control group.

control	age categories groups			*P* value
rs6166	≤15year (n=4)	16-19year (n=7)	≥20year (n=29)	0.47
AA	1 (7.69%)	4 (30.77%)	8 (61.54%)	
AG	3 (16.67%)	2 (11.11%)	13 (72.22%)	
GG	0 (0%)	3 (25%)	9 (75%)	

Though PA cases as well divided to the same categories of ages, ≤15year(n=18) with genotype distribution (46.66%), (31.82%)and (21.05%) for AA, AG and GG respectively, while 16-19year (n=17) with (26.67%), (27.27%) and (36.84%) for AA, AG and GG respectively and the third category with ≥20year (n=21) with (26.67%), (40.91%) and (42.11%)for genotypes AA, AG and GG, as illustrated in table(5B). The results showed no significant correlation between genotype and age for control and PA groups. with p=(0.47) and p=(0.72) respectively.

Table(5B): rs6166 *FSHR* genotypes frequencies and age categories in the PA group

PA	age categories groups			*P*-value
rs6166	≤15year (n=18)	16-19year (n=17)	≥20year (n=21)	0.72
AA	7 (46.66%)	4 (26.67%)	4 (26.67%)	
AG	7 (31.82%)	6 (27.27%)	9 (40.91%)	
GG	4 (21.05%)	7 (36.84%)	8 (42.11%)	

It is clear from all results previously displayed done by ANOVA statistical analysis, there is no relation of genotype change with different levels of serum hormones, that is so because our SNP do not have grate impact on *FSHR* expression and functionality of proteins translated, although the SNP belongs to non-synonymous nucleotide change but until now there is no evidence that it could effects on receptor action, as well the effect of SNP of interest with FSH level could depends on ethnicity and populations , Kuijper *et al.* (2010) in which they studied about relation of FSH with rs6166 according to ethnicity and found that FSH level of infertile females of Asian population have lower significant alteration compared with Caucasians and Mediterranean and also they discovered no significant link between genotypes and levels of FSH, LH, or E2 in the fertile and infertile groups, nor between the frequency of alleles and genotypes of codon 680 in exon 10. Another study on primary amenorrhea cases Achrekar and Modi, (2010) showed that FSH level in serum

affected significantly only by a polymorphism in the promoter region of *FSHR* gene, that showed a high level of S.FSH related with PA cases. Sudo *et al.*(2002) reported that in the population of Japanese women, there are no significant differences in genotype distribution and basal FSH.

In the present study, results also showed that there is no association between ages of control and PA groups with genotype variation, this results confirmed by other studies such Ilgaz *et al.* (2015) and Genzlinger, (2013) in which they studied on group of women suffering from premature ovarian failure(POF) and found that there is no significant relation with age. According to a study done on patients with polycystic ovary syndrome(PCOS) Kim *et al.* (2017), results showed no significant association of age and gonadal hormones with genotype variation with p= 0.398, p= 0.08 and p= 0.391 correspondingly. All results discussed above confirmed that all these female reproductive problems PA, PCOS, infertility, and POF have no association of hormonal alteration when compared with rs6166 genotype variation.

primary amenorrhea problem related with many other outcomes such as infertility and premature ovarian failure and these problems in the present years could be solved throughout many ways such as IVF and COH programs, all these solve ways depends recently on molecular analysis results of SNPs and genotypes variation of these variants. According to Achrekar *et al.*(2010), they studied on the impact of Thr307Ala with ovarian stimulation program which could help females with fertile and reproductive outcomes, as well they showed that basal FSH and LH not effected with genotype variation and also found that the patients need much more exogenous FSH with mutant genotype rather than heterozygote and wild genotype. Ilgaz *et al.* (2015) studied on the impact of FSHR polymorphisms on a group of infertile women in a case-control study, results showed no significant differences between the endocrine profile and genotype distribution but showed that mean± SD value increase with mutant genotype and decrease with reference and heterozygote genotypes. Meng *et al.* (2018) studied also on a group of a female under COH program and found that there is no association between gonadal hormones and rs6165 polymorphism. Besides they found the mean of hormones rises with mutant genotype and finally concluded that they needs much more Gn dose in a treatment program. Yan *et al.* (2013) showed that there is a strong association of hormones with genotype variation especially in AA and AG rather than GG, which agreed with present study findings.

Conclusion

The current study identify that the *FSHR* gene polymorphism rs6166 not support the genetic predisposition of primary amenorrhea, but have an impact on the hormonal levels, that may have a strong role in secondary amenorrhea.

References

1. Achrekar, S. K., Modi, D. N., Meherji, P. K., Patel, Z. M. and Mahale, S. D. (2010). Follicle stimulating hormone receptor gene variants in women with primary and secondary amenorrhea. Journal of Assisted Reproduction and Genetics, 27(6), 317–326.
2. Hammond, D. A., Olman, V. and XU, Y. (2014). Functional Understanding of the Diverse Exon–Intron Structures of Human Gpcr Genes. Bioinformatics and Computational Biology, 12(01), 1350019.
3. Ilgaz, N. S., Sena, O., Aydos, E., Karadag, A., Taspinar, M., Eryilmaz, O. G. and Sunguroglu, A. (2015). Impact of follicle-stimulating hormone receptor variants in female infertility. 1659–1668.
4. Kim, J. J., Choi, Y. M., Hong, M. A., Chae, S. J., Hwang, K., Yoon, S. H., and Kim, S. H. (2017). FSH receptor gene p. Thr307Ala and p. Asn680Ser polymorphisms are associated with the risk of polycystic ovary syndrome. Journal of Assisted Reproduction and Genetics, 34(8), 1087–1093.
5. Kiyoi, H., Naoe, T., Yokota, S., Nakao, M., Minami, S. and Kuriyama, K. (1997). Internal tandem duplication of FLT3 associated with leukocytosis in acute promyelocytic leukemia. Leukemia, 11:1447– 1452.
6. Kuijper, E. A. M., Blankenstein, M. A., Luttikhof, L. J. and Roek, S. J. M. (2010). Frequency distribution of polymorphisms in the FSH receptor gene in infertility patients of different ethnicity. Reproductive BioMedicine Online, 22(5), S60–S65.
7. Lindgren, I (2017). "The impact of gonadotropin receptor polymorphisms on human reproductive function", Doctor, Department of Translational Medicine, L.
8. Meng, Q., Song, D., Li, H., Wang, W., Ou, J., Xu, Y. and Zheng, A. (2018). Association of Thr307Ala and Asn680Ser of Follicle - Stimulating Hormone Receptor Gene Polymorphisms with Gonadotropin Administration during Controlled Ovarian Hyperstimulation. 2(2), 81–87.
9. Mu, Q., Hanson, L., Hoelzle, J. and Fehring, R. J. (2019). Young Women's Knowledge About Fertility and Their Fertility Health Risk Factors. JOGNN - Journal of Obstetric, Gynecologic, and Neonatal Nursing, 48(2), 153–162.
10. Nawaz G, R. A. (2019). Amenorrhea. In StatPearls [Internet].
11. Reed G. (2018). The Normal Menstrual Cycle and the Control of Ovulation. Endotext. South Dartmouth
12. Samal, R. and Habeebullah, S. (2017) 'Primary amenorrhea: a clinical review', International Journal of Reproduction, Contraception, Obstetrics and Gynecology, 6(11), p. 4748. doi: 10.18203/2320-1770.ijrcog20174981.
13. Simoni, M. and Casarini, L. (2014). Mechanisms in endocrinology: Genetics of FSH action: a 2014-and-beyond view. European Journal of Endocrinology, 170(3), R91–R107.
14. Sudo, Satoko, Kudo, M., Wada, S., Sato, O., Hsueh, A. J. W. and Fujimoto, S. (2002). Genetic and functional analyses of polymorphisms in the human FSH receptor gene. 8(10), 893–899
15. Thomas, M., Srivastava, S. and Devi, R. (2014). Presence of the ASN680SER Polymorphism in women with Primary Amenorrhea from South India. (June), 358–367.
16. Ulloa-aguirre, A., Zariñán, T., Jardón-valadez, E., Gutiérrez-sagal, R., Dias, J. A. and Stanton, P. (2018). Structure-Function Relationships of the Follicle-Stimulating Hormone Receptor.1–17.
17. Van der Wijden, C. and Manion, C. (2015). Lactational amenorrhoea method for family planning. Cochrane Database of Systematic Reviews, 2015(10).

18. Yan, Y., Gong, Z., Zhang, L., Li, Y., Li, X., Zhu, L. and Sun, L. (2013). Association of Follicle-Stimulating Hormone Receptor Polymorphisms with Ovarian Response in Chinese Women : A Prospective Clinical Study. 8(10), 1–8.
19. Zaker, F., Mohammadzadeh, M. and Mohammadi, M. (2010). Detection of KIT and FLT3 mutations in acute myeloid leukemia with different subtypes. Arch. Iran Med., 13(1):21