ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Molecular analysis of prolactin gene exons region of hyperprolactinemic and breast cancer Iraqi patients

***Marwa Kubba[1], Abdulwahid Jabir[2] and Rehab Ramadan**

1. Department of Biotechnology, Al-Nahrain University, Baghdad.
2. Department of Biotechnology.

Manuscript Info

Manuscript History:

Received: 14 December 2015
Final Accepted: 16 January 2016
Published Online: February 2016

Key words:
Prolactin,
Hyperprolactinemia,
Breast cancer,
Mutation

****Corresponding Author***

Marwa Kubba.

Abstract

This study was constructed to investigate hyperprolactinemia related infertility through the molecular base associated with single nucleotide polymorphism (SNP) at prolactin gene in hyperprolactemic patients with the breast cancer risk**.** This study included one hundred fifty blood samples from patients suffering hyperprolactinemia and infertility. Also twenty five tissue samples of breast cancer patients were collected in which fifteen samples were frozen tissue and ten samples were formalin fixed paraffin embedded tissue. Fifty blood samples from healthy persons were collected served as control group. The main ages of patients were 20 to 50 and same for control (healthy) group. it's clear that there is substitution and deletion mutation, in which the highest mutation number was in exon 2, which was 9 mutations, all mutations in this exon was substitution ,while the less mutation number was in exon 3 and exon4 which was 2 for each exon, one substitution and one deletion mutation in exon 3 while the two mutations in exon 4 was substitution only. The risk association between the DNA of hyperprolactemic and breast cancer patients using information on national center for biotechnology information (NCBI), and Mega 6 program.

Introduction:-

Prolactin (PRL) is a polypeptide hormone of a pituitary origin, whose production is controlled by dopamine and this hormone have many biological activity such as lactation and reproductive functions [Bernichtein et al.,2010]. Hyperprolactinemia is a condition of presence of abnormally high level of prolactin in the blood in which normal levels are 10-21 mU/ml, this condition present as a pathological condition [Davis ., 2004].

Hyperprolactinemia represent a common problem in reproductive dysfunction, in which it lead to high circulating levels of prolactin and hypogonadism which lead to lack of gonadotrophin cyclicity and to infertility, The human prolactin gene is present as a single copy on chromosome 6 it is about 12.215 kb) contains 5 exons and 4 introns and the transcription of it is regulated by two promoters is used in extra pituitary cells and tissues and downstream promoter that directs transcription in pituitary lactotrophs [Rui H and Nevalainen MT., 2000]. As the prolactin is an essential regulator of mammary development, the primary cells targeted by prolactin are the breast tissue cells in which it is involved in the development of mammary gland and in cellular growth and differentiation as well as in the initiation and maintenance of lactation [Mong et al., 2011]. studies demonstrated that prolactin could induce spontaneous mammary tumors and can stimulate proliferation [Liby et al., 2003].

Material and Methods:-

- **Patients selection and blood sample collection:-**
- Blood sample were collected from one hundred fifty (150), infertile women suffering from hyperprolactinemia their ages ranged from (20-50) years, and 50 blood samples collected from healthy samples. Samples were subjected to centrifugation at2000 rpm for 10 min. The serum was separated and stored at -20^{0}C. All samples were subjected for biochemical analysis. In case of blood with EDTA, it was stored at -20 C until used for DNA extraction. The samples were obtained from infertility hospital Kamal Al-Sammaraee. The collection period extended from march 2014 to September 2014. Also a total of twenty five tissue samples, in which 10 paraffinzed breast tissue samples and 15 frozen tissue with breast cancer was obtained from Al-Khadmyaa teaching hospital. Total cellular DNA was extracted from blood samples by using the reliaprep blood genomic DNA MiniPrep System from Promega USA estimation the concentration and purity of the extracted DNA were measured by using nanodrop (UVIS Drop\ACTGene\USA). Primers

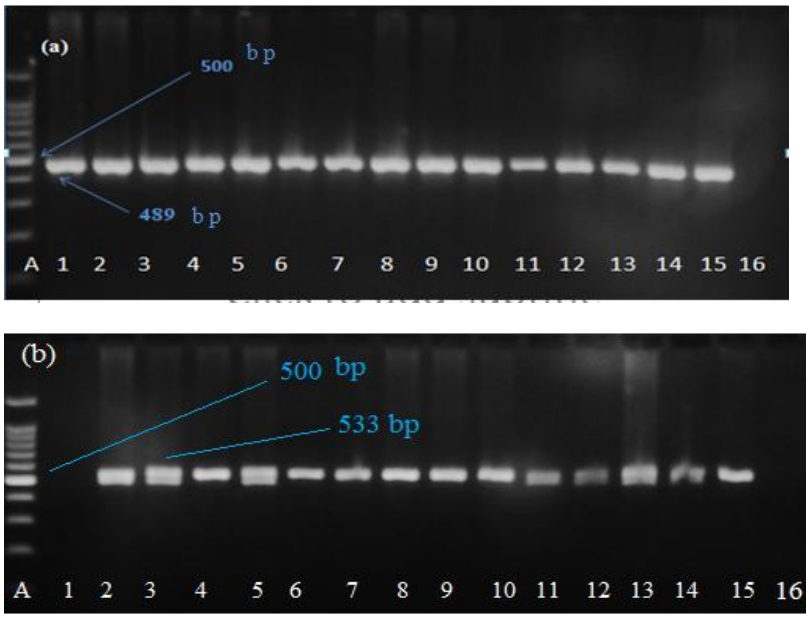
NO	OLIGONUCLEOTIDE	OLIGOSEQUNESE	PROD.SIZE (BP)	GC%	TM	REF.
1	FORWARD PRIMER REVERSE PRIMER	ATGTGTGACAACTCACTGCG GGCCAATCCACATTAGAGGC	489	50.00 55.00	51.78 53.83	NCBI
2	FORWARD PRIMER REVERSE PRIMER	GCTGAATCCATGGTGGGGAA TCTCTGTGGAGGCCCTTGAT	533	55.00 55.00	53.83 53.83	NCBI
3	FORWARD PRIMER REVERSE PRIMER	AAACGGTATACCCATGGCCG AGTGGCAACTGTAGCTGTGA	719	50.00 55.00	53. 83 51.78	NCBI

- **PCR amplification**
  the preparation of PCR reaction mixed on ice and was carried out in 25 µl of Go Taq Green masrer mix . The amplification condition were as following for primer 1, initial denaturation 94^{0}C for 5 min, denaturation 94^{0}C for 1 min, annealing 1 min at 55^{0}C, extension 1 min at 72^{0}C for 35 cycles, and final extension 72 ^{0}C for 10 min. Same program used for amplification of prolactin gene using primer 2 and 3, but with different annealing temperature, in which its 57^{0}C for 1min by sing primer2 and 59^0 C for 1 min by using primer 3.
- **DNA gel electrophoresis :** the quality of extracted DNA and PCR amplicons was checked with 1% agarose gels at 90V for 90 min.
- **Statistical analysis:** the statistical package for the social sciences (ANOVA version 15).

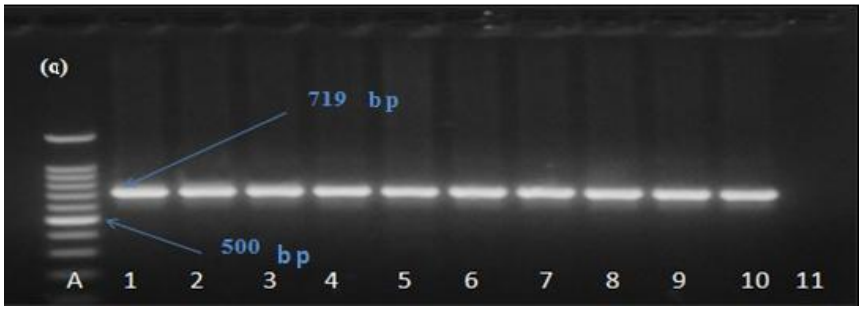
Result and Discussion:-

Prolactin gene amplifications.:-

Primers were used in this PCR technique(PRL1, PRL2, PRL3)specific to amplify exons region of prolactin gene. As it can be seen in the figures below the sizes of PCR products relevant to each exon was primer 1, product size 489 bp for exon 2, primer 2, product size 533 bp for exon 3, and primer 3, product size 719bp for exon 4. Figures (1), (2) showed the PCR amplifications of some of hyperprolactemic and breast cancer patients, also control samples that used in this study.



Figure(1a, b): Gel electrophoresis for amplification of PRL gene of hyperprolactinemic patients by using (a):primer 1 which amplifies exon 2 of the gene, product size 489 bp. (b): primer 2 which amplifies exon 3 of the gene, product size 533bp. Electrophoresis was performed on 1.5% agarose gel and run with a 80v/mAMP current for 50min.Line A=100bp ladder, line(1-5) DNA isolated from blood samples of hyperprolactinemic patients, line (6 -10)DNA from frozen tissue, line (11-14) DNA isolated from FFBE tissue, line (15) healthy ,line 16 control negative



Figure(2): Gel electrophoresis for amplification of PRL gene of hyperprolactinemic patients by using primer 3 which amplify exon 4 of the gene, product size 719 bp. Electrophoresis was performed on 1.5% agarose gel and run with a 80v/mAMP current for 50min.Line A=100bp ladder, line(1-3) DNA isolated from blood samples of hyperprolactinemic patients, line (4-6)DNA from frozen tissue, line (7-9) DNA isolated from FFBE tissue, line (10) healthy , line(11) control negative

Detection of PRL mutations in hyperprolactemic and breast cancer patients by sequencing:-

After amplification of genomic fragments corresponding exon 2 to exon 4 of the PRL gene. The PCR products (489, 533,719 bp) for prolactin gene good as shown in figures. By using the DNA of above cases good quality products (pure and concentrate) were selected to be sequenced. To evaluate if any genetic variation in the PRL and PRL receptor gene as predictors of high prolactin levels and breast cancer risk.

The sequencing was done to infected women which is 15 sample for each exon of hyperprolactinemic patient and 10 for breast cancer patients, 5 for control, The sequence involved part of the PRL gene spanning from nucleotide number6169 to nucleotide number 17680 of the chromosome 6.The results were directly compared with the Iraqi healthy, and compared with the data obtained from the gene bank published by BLAST program which is available at the NCBI online at www.ncbi.nlm.nih.gov and also by using Mega 6 program. The current study

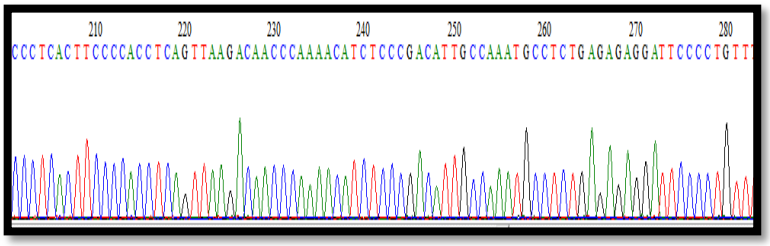
utilized forward and reverse primer for sequencing PRL gene of blood and tissue sample of hyperprolactemic and breast cancer patients. It was found that mutations were found in around all PRL gene regions involved in this study, which is (exon2, exon3, exon4), and according to NCBI this streach contain 13 SNPs.

The mutation frequency was different among the three studied region of the gene. the highest mutation number was in exon 2, which was 9 mutations while the less mutation number was in exon 3 and exon4 which was 2 for each exon.

Detection of mutations in PRL gene:-

The polymorphisms that we observed within the exon 2 of PRL gene are shown in figure (3), appear patients that have polymorphisms with the healthy samples, all polymorphisms in this exon observed in infertile hyperprolactemic patients while no mutation detected in breast cancer hyperprolactemic patients. The substitutions are obvious in the figure (3 a, b). A homology with the PRL gene of *Homo sapiens* from the Gene Bank was done , 100% compatibility of that gene of healthy samples with standard genes of Gene Bank results as shown in figure (4 a, b).

(a)



(b)

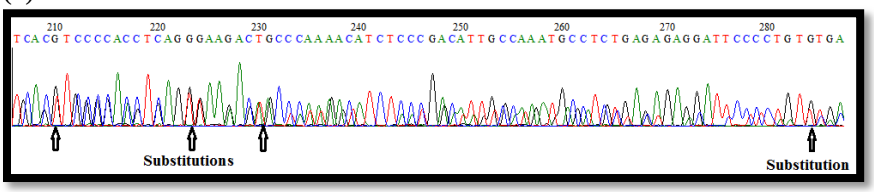
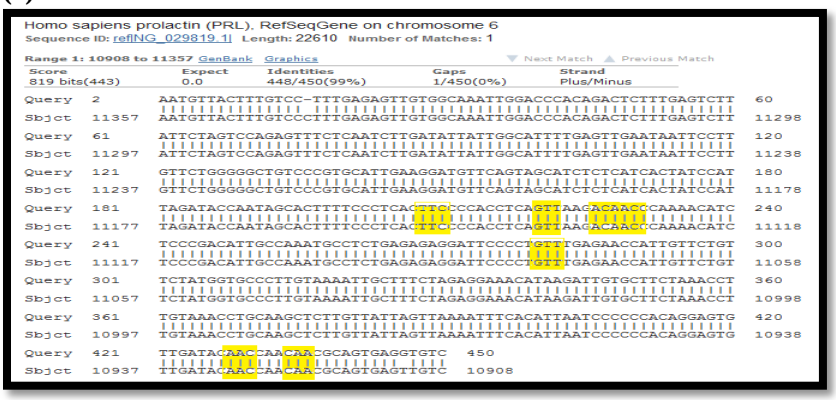


Figure (3) a and b. Achromatogram comparision between substitutions SNPs and control for Exon 2.

(a)



(b)

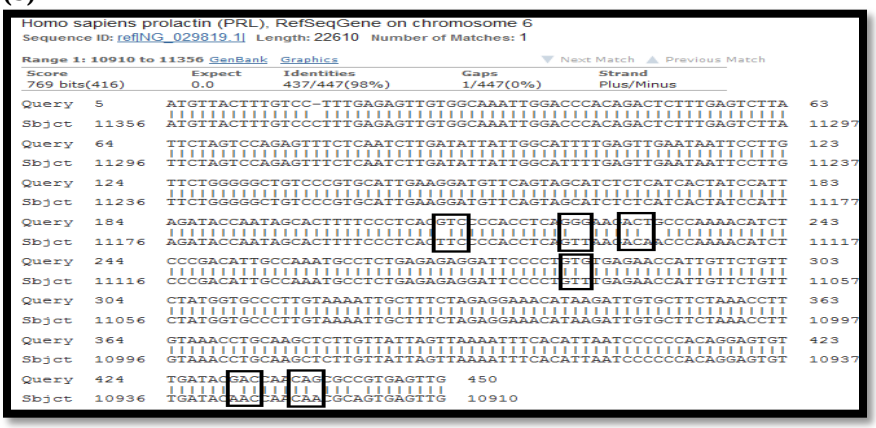
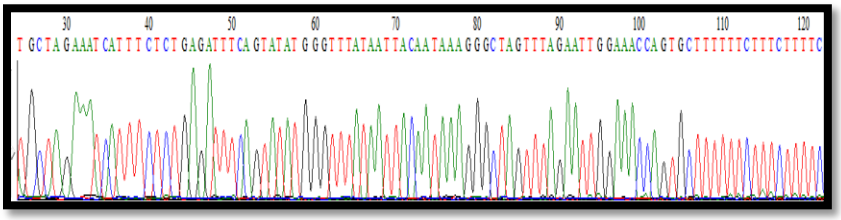


Figure (4) a and b: The automated sequencing of the exon 2 of PRL gene A: of healthy samples, B: for hyperprolactemic patient.

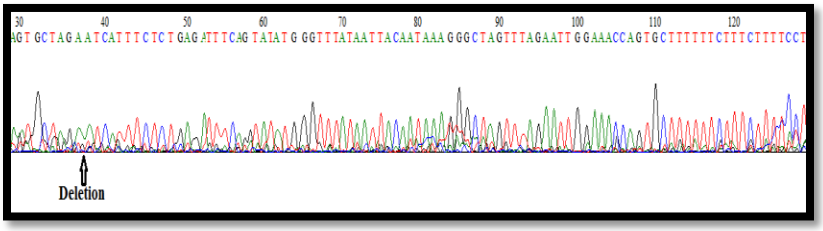
Representation of the sample by query and the subject represents of database of national Center Biotechnology Information.

In this region of the gene of hyperprolactemic patients, there is many SNPs, 7 SNPs in sample 1 which is TTC/GTC, in position 210 that convert a.a Phe to Val, the other GTT/GGG in position 223 and 224, that convert a.a Val to Gly, also in position 230 the ACA/ ACT which convert a.a Thr to Thr, but in position 231 the ACC changed to GCC that convert a.a Thr/Ala. In 284 the GTT converts to GTG which convert a.a Val/ Val. The last two SNPs in this sample that is common with sample 4 is , AAC/GAC in position 438, that convert a.a Asp/Asp, and the other common SNP between two samples is CAA/CAG which convert a.a Gin/Gin. In the same region of exon , but in sample 3 of hyperprolactemic patient there is 2 SNPs, first in position 363 in which ATT convert to GTT, that convert Ile/ Val, and the other one in position 400 TAA/TGA that convert Stop/Stop. The peaks that appear the mutations are clear in figure (5) a, b and c. The homology of this region of exon with the blast of NCBI was obvious in figure (6) a and b.

(a)



(b)



(c)

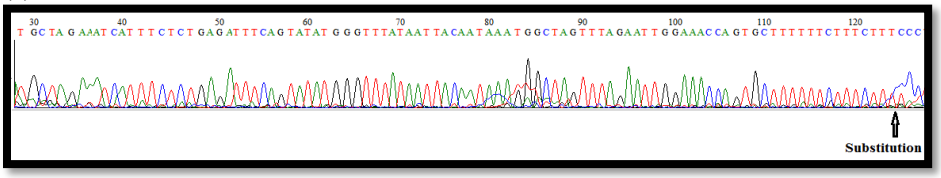
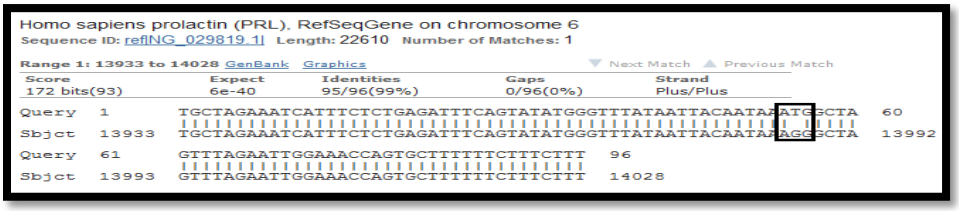


Figure (5) a, b and c: Achromatogram of (a) control, (b) breast cancer patient (c) hyperprolactemic patient, of exon 3 amplified by using primer 5.

(a)



(b)

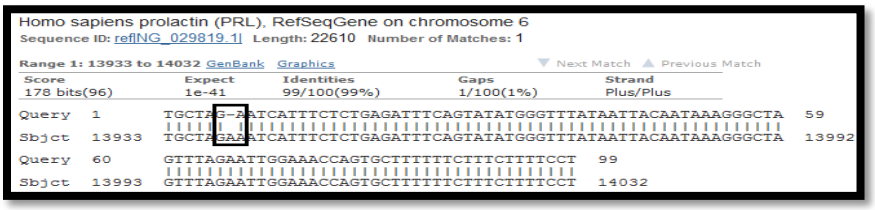


Figure (6) a and b :Sequencing of exon 3 of PRL gene a: of hyperprolactemic patient, b: for breast cancer patient

The two SNPs in this region of the gene is AGG/ATG in position 125 of hyperprolactemic patient which convert Arg to Met, the other in breast cancer sample which is GAA convert to G-A in position 36 that convert a.a Glu to deletionThe other exon that we examined is exon 4 by using primer 6, and its shown that there is heterozygous SNPs in two samples of breast cancer, which is in position 290, the peaks as its shown in figure(7), it's obvious in NCBI, figure (8) and also there is a substitution SNP s in all samples hyperprolactemic and in breast cancer in position 379, TGA to TGG that convert a.a Stop/Trp.

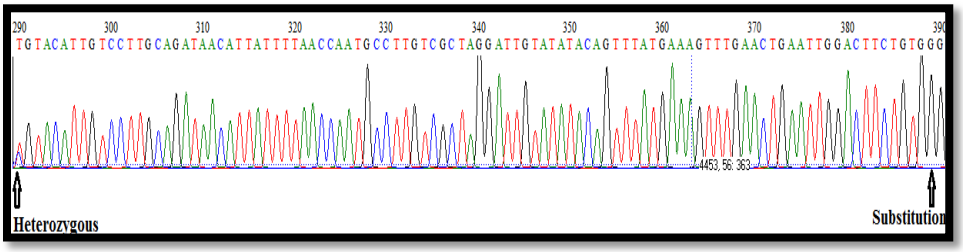


Figure (7). Heterozygous and substitution SNPs of breast cancer patient .

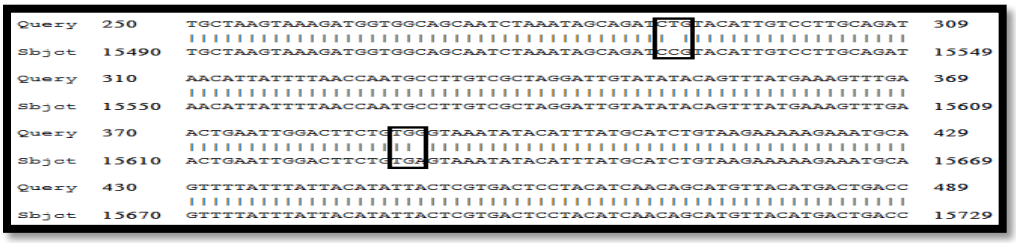


Figure (8): Sequencing of exon 4 of PRL gene for breast cancer patient illustrated the substitution and heterozygous mutations

While show the table (1), different mutation of one or more than located gene region. However point mutation , substitution , deletion affected the PRL gene in Iraqi patients

Table (1): Mutation types of human PRL gene in both hyperprolactemic and breast cancer patients

DISEASE TYPE	SITE ON GENE	WILD TYPE	MUTANT TYPE	CHANGE IN A.A	SITE ON NUCLEIC ACID	TYPE OF MUTATION
HYPERPROLACTINEMIA	EXON 2	TTC	GTC	PHE/VAL	210	SUBSTITUTION
		GTT	GGG	VAL/GLY	223,224	SUBSTITUTION
		ACA	ACT	THR/THR	230	SUBSTITUTION
		ACC	GCC	THR/ALA	231	SUBSTITUTION
		GTT	GTG	VAL/VAL	284	SUBSTITUTION
		ATT	GTT	ILE/VAL	363	SUBSTITUTION
		TAA	TGA	STOP/STOP	400	SUBSTITUTION
		AAC	GAC	ASP/ASP	438	SUBSTITUTION
		CAA	CAG	GIN/GIN	446	SUBSTITUTION
BREAST CANCER	EXON 3	GAA	G-A	GLU-DELETION	36	DELETION
HYPERPROLACTINEMIA		AGG	ATG	ARG/MET	125	SUBSTITUTION
HYPERPROLACTIEMIA	EXON 4	TGA	TGG	STOP/TRP	389	SUBSTITUTION
		TGA	TGG	STOP/TRP	389	
		TGA	TGG	STOP/TRP	389	
		TGA	TGG	STOP/TRP	389	
		TGA	TGG	STOP/TRP	389	
BREAST CANCER		TGA	TGG	STOP/TRP	389	
		TGA	TGG	STOP/TRP	389	
		TGA	TGG	STOP/TRP	389	
		TGA	TGG	STOP/TRP	389	
		TGA	TGG	STOP/TRP	389	
		TAT	TTT	TYR/PHE	287	SUBSTITUTION

Genetic factors are important possibly for the disease in many samples of patients, but its not clear which region of the gene contributes exactly to disease. The exons and introns region of the PRL gene shows mutations in hyperprolactemic and breast cancer patients and some region of the gene shows a common mutation in some bases in both diseases of some patient samples. The mutations that detected in exons region of PRL gene of hyperprolactemic patients give a evidence that this mutations play a part in this disease As there is increasing evidence that PRL gene is involved in the development of breast cancer, it was found that SNPs in the exons and introns of the PRL gene was detected and this polymorphisms alter expression attributable to altered transcription factor gene binding.

This result was same in study by Vaclavicek *et al*., 2006 who detected a new SNP in PRL gene who showed that a significant association between promoter SNPs (G/T) and (A/G) of PRL gene and breast cancer in which this effect was carried by the TGTG haplotype which was significantly associated with an increased BC risk.[Delgrange et al., 1999]. Also a rare homozygous genotypes of the (A/T) SNP near exon 2 and the (G/A) SNP near exon 5 more frequent in the patients than in controls. But in same study the existence of a (Arg/Stop) SNP, in exon 4 of PRL gene could not be confirmed after sequencing a 96 breast cancer sample , while in our study a two heterozygous SNP was detected (Stop/Trp) in two BC samples.

From our study it was shown that there is mutation in PRL gene and some mutation common with breast cancer patients, so this association was detected between the PRL gene mutation and breast cancer.

This has most extensively in connection of hyperprolactinemia with breast cancer as , Mong et al., (2011) , when he carried out an association study and first confirmed that the SNP in PRL gene is strongly associated with metastasis of breast cancer in Taiwanes subjects [Mong et al., 2011].

In our study there is SNPs in hyperprolactemic patients and same in breast cancer patients in same bases in exon 2 and exon 4, this may be because patients with hyperprolactinemia might be exposed to high levels of PRL for several years either because of delay of dignoses or treatment failure[Ben-Jonathan et al., 2002], and PRL is involoved in mammay gland growth and differentiation so the overexpressing of prolactin as which happened in hyperprolactinemia patients will correletes with increased mammary tumorigenesis[Faupel-Badger et al., 2011]. This agree with study by Faupel-Badger et al., (2010)[Lee et al., 2007]. Who reported that higher prolactin levels was associated with increased breast cancer risk. Also agree with Lee et al., 1997, who discovered a low frequency synonymous SNP in exon 3 (A/G) also in exon 5, but not in exon 2, and also a missense SNP in exon 4 when he made a comprehensive analysis of common genetic variation in PRL and PRLR genes in relation to plasma prolactin levels and breast cancer risk. This hormone PRL physiological influences the mammary gland in several ways during development, growth and stimulation of milk protein gene transcription [Neville et al., 2011]. Also the importance of PRL in pathological conditions such as mammary tumor growth in which PRLR has been formed in 40-70% of human breast tumors and PRL stimulate growth of several human breast cancer cell lines invitro indicating a possible auto/paracrine function of PRL in many tumor growth. The role of it on breast cell proliferation is the tumor growth promoting effect of PRL signaling in the mammary gland are well documented in animal model [Ormandy et al., 2003, Nitze et al., 2013]. This results is also agreement with Nitze et al., (2013), [Vyas and Risk 2012], who found that as prolactin has been implicated in tumorgenesis, its important for proliferation and differentiation of the breast epithelium and its shown that PRL and its receptor are co-expressed in breast cancer tissue and cell lines and thus PRL has been suggested to promote growth of the carcinomas in autocrine/paracrine fashion [8].The studies that linked hyperprolactinemia with increased risk of breast cancer is the mechanisms that have been suggested to explain this possible action of prolactin include the increased synthesis and expression of prolactin receptors in malignant breast tissue and prolactin induced increase in DNA synthesis in breast cancer cell invivo [Plotnikov et al., 2009].

And is agreement with study of Plutnikov, (2009),[Nore et al., 2013], who shows that as the PRL its signaling is mediated by its congate receptor, so prolactin receptor is commonly stabilized in human breast cancer due to decrease in phosphorylation of residue serum which when phosphorylated facilities PRLR degradation so the import PRLR turnover result in augmented PRL signaling and PRL induced transcription.

In the present study it was found that in addition to mutations that are detected in exon region of the PRL gene of infertile hyperprolactemic patients, there are also many mutations in intron region of the gene as its clear in the above figures the mutations in intron 1, and this consist with the result that obtained by the Iraqi study as regards that mutations in intron 1 and 2 of prolactin gene of infertile hyperprolactinemic women[Vaclavicek, et al., 2006]. Who mentioned the mutations in hyperprolactinemic patients in intron region of the PRL gene, and thus considered as genetic marker for high prolactin level causing infertility in Iraqi women. Also it was found that there is mutations in breast cancer patients in the same region of the gene which make the association of the mutation in this region of the gene, hyperprolactinemia and breast cancer clear was shown a significant association between the promoter SNPs of the prolactin gene and breast cancer. Positive results for the association of PRL polymorphism in hyperprolactemic patients and breast cancer disease as the same SNP were found in the same position of both disease and this result was same.

References :-

1. **Bernichtein, S., Touraine, P., Goffin, V.** New concepts in prolactin biology. Journal of Endocrinology. 2010; 206(1), 1-11.
2. **Davis, J.R**., . Prolactin and reproductive medicine. Current Opinion in Obstetrics and Gynecology, 2004; 16(4), 331-337.
3. **Rui, H., Nevalainen, M.T.**, Prolactin. Oppenheim JJ, Feldman M Cytokine reference on-line. London, UK: Academic Press, Harcourt, 2000; 267-283.
4. **Mong, F.Y., Kuo, Y.L., Liu, C.W., Liu, W.S. Chang, L.C.** Association of gene polymorphisms in prolactin and its receptor with breast cancer risk in Taiwanese women. Molecular biology reports. 2011; 38(7), 4629-4636.

5. **Liby, K., Neltner, B., Mohamet, L., Menchen, L., Ben-Jonathan, N.,** Prolactin overexpression by MDA-MB-435 human breast cancer cells accelerates tumor growth. Breast cancer research and treatment, 2003; 79(2), 241-252.
6. **Delgrange, E., Trouillas, J., Maiter, D., Donckier, J., Tourniaire, J.** Sex-Related Difference in the Growth of Prolactinomas: A Clinical and Proliferation Marker Study 1. The Journal of Clinical Endocrinology and Metabolism, 1997; *82*(7), 2102-2107.
7. **Ben-Jonathan, N., Liby, K., McFarland, M.** . Prolactin as an autocrine/paracrine growth factor in human cancer. Trends. Endocrinol Metab. 2002; 13:245–250.
8. **Faupel-Badger, J.M., Sherman, M.E., Garcia-Closas, M., Gaudet, M. M., Falk, R.T., Andaya, A., Figueroa, J.D.** Prolactin serum levels and breast cancer: relationships with risk factors and tumour characteristics among pre-and postmenopausal women in a population-based case–control study from Poland. British journal of cancer. 2010; 103(7), 1097-1102.
9. **Lee, S.A., Haiman, C.A., Burtt, N.P., Pooler, L.C., Cheng, I., Kolonel, L.N., Pike, M.C., Altshulter, D., Hirs Chhorn, J.N., Henderson, B.E., Stiam, D. O.2007;** A comprehensive analysis of common genetic variation in prolactin PRL and PRL receptor genes in relation to plasma prolactin levels and breast cancer risk : the multiethnic cohort.BC Med Genet. 1997; 8: 72.
10. **Wennbo, H., Gebre-Medhin, M., Gritli-Linde, A., Ohlsson, C., Isaksson, O.G., Törnell, J.,** Activation of the prolactin receptor, but not the growth hormone receptor is important for induction of mammary tumors in transgenic mice. Journal of Clinical Investigation, 1997; 100(11), 2744-2751
11. **Neville, M.C., McFadden, T.B., Forsyth, I.,** Hormonal regulation of mammary differentiation and milk secretion. Journal of mammary gland biology and neoplasia. 2002; 7(1), 49-66.
12. **Ormandy, C.J., Naylor, M., Harris, J., Robertson, F., Horseman, N.D., Lindeman, G.J., Kelly, P.A..** Investigation of the transcriptional changes underlying functional defects in the mammary glands of prolactin receptor knockout mice. Recent Progress in Hormone Research, 2003; 58, 297-324.
13. **Nitze, L.M., Galsgaard, E.D., Din, N., Lund, V.L.,** Rasmussen BB, Berchtold MW, Panina S. Reevaluation of the proposed autocrine proliferative function of prolactin in breast cancer. Breast cancer research and treatment, 2013; 142(1), 31-44.
14. **Vyas, U.,** Risk of breast cancer due to hyperprolactinemia caused by antipsychotics (neuroleptics). British Journal of Medical Practitioners, 2012; 5(4).
15. **Plotnikov, A. Varghese, B. Tran, T.H., Liu, C. Rui, H.** Fuchs SY Impaired turnover of prolactin receptor contributes to transformation of human breast cells. Cancer research, 2009. 69(7), 3165-3172.
16. **Nore**, **F.B., Mahmoud, T.J., Rashid, B.M.,** . Sequence verifications and promoter analysis of the prolactin gene. Journal of Zankoy Sulaimani. 2013; 15(1):1.
17. **Vaclavicek, A. Hemminki, K, Bartram, C.R., Wagner, K., Wappenschmidt, B., Meindl, A., Schmutzler, R.K., Klaes, R., Untch, M., Burwinkel, B., Forsti, A. (2006)**. Association of prolactin and its receptor gene regions with familial breast cancer. Jol.Clinical. Endocrinology and metabolism:91(4):1513-1519.
18. **Al-Katanani, Y.M., Paula Lopes, F.F. and Hansen, P.J. (2002).** Effect of season and exposure to heat stress on oocyte quality of Holstein cows. J. Daiy Sci. 58: 171-182.
19. **Butler, W.R. (2001).** Nutritional effects on resumption of ovarian cyclicity and conception rate in postpartum dairy cows. In Diskin M.G. (ed.).
20. **Dhoble, R.L. and Guplta, S.K. (1981).**Biochemical parameters and response to gonadotrophin administration in anoestrus buffaloes. Indian J. Anim. Sci. 57:47-50.
21. **Hala, A.A., Abou-Zeina, Hassan, S.G., Sabra, H.A. and Haman, A.M. (2009).** Trials for elevating adverse effect of heat stess in buffaloes with emphasis on metabolic status and fertility. Global Veterinaria 3(1): 51-62.
22. **Mohammed, F.C., Dhaliwal, G.S. and Sharma, R.K. (1999).** Clinical efficacy of GnRH analogue (Buserelin) and oestradiol benzoate treatment in anoestrus buffaloes. Indian J. Anim. Sci. 69(5): 310-312.1