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(RESEARCH ARTICLE)

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Genetic detection of prolactin intron 1 region in breast cancer patients of Iraqi women

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Abstract

This study was made to inspect the genetic cause of breast cancer through the molecular base related with single nucleotide polymorphism (SNP) at prolactin gene in patients with the breast cancer .This study comprised thirty blood samples from patients suffering from breast cancer. Also thirty tissue samples of breast cancer patients were collected in which samples were formalin fixed paraffin embedded tissue. thirty blood samples from healthy persons were collected served as control group. The main ages of patients were 19 to 60 and same for control (healthy) group.The variation in gene that accountable for synthesis of hormone prolactin, was conducting using samples of breast cancer patients to demonstrate if this variation is important to breast cancer risk In addition to. Polymerase chain reaction (PCR), was done by using specific set of primers , in which 3 primers were selected to amplify the intron 1 region of the gene.

After optimization of the amplification condition, the product was sent for DNA sequencing for detection of variation of patient prolactin gene, so the association of this variation of prolactin gene to breast cancer patients was clear after studying the intron region. In intron 1 of the gene, 7 mutation was detected by using primer 1, in which two of them is deletion mutation and 5 was substitution, while in same intron of the gene but using primer 2, 6 mutation was detected all is substitution. The risk association between the prolactin intron gene association and breast cancer patients using information on national center for biotechnology information (NCBI), and Mega 6 program.The results of mutation detection in the PRL gene intron region showed that there is occurrence of mutations in of breast cancer patients samples

Keywords: Single nucleotide polymorphism; Breast cancer; Prolactin intron 1 gene

1. Introduction

Prolactin (PRL) is a polypeptide hormone of a pituitary origin, whose production is organized by dopamine and this hormone have many biological activity such as lactation and reproductive functions (Bernichtein *et al*., 2010).

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 and Nevalainen, 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).

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A woman's genetic background contributes to her risk of having breast cancer because the risk of developing breast cancer increases in women if she has a family history of the disease (Lallo and Evans, 2012).

About 90 genes or genetic loci are involved in breast cancer susceptibility in general this was through rare, moderate to high penetrance mutations (lifetime risk >20%). The penetrance being the risk of a mutation carrier of developing a disease, or through common variants associated with risks that are only slightly increased compared to the wild-type allele (Couch *et al.,* 2014). In most cases the genetic variation plays a role and it is thought that genetics is the primary cause of 5-10% of cases (Gage et al., 2012).

Molecular oncology is now one of the greatest hopeful fields that may contribute significantly to diagnosis of breast cancer and its metastases address major problems with early detection, accurate staging, and monitoring of breast cancer patients.

The linkage between variation of both genes of PRL and PRLR with breast cancer was detected (Canbay et al., 2004; and Lee et al., 2007). Furthermore, this variation in these genes was shown to effect on breast cancer risk (Vaclavicek *et al*., 2006) because it was expressed in both normal and malignant breast tissues (Ben-Jonathan *et al*., 2002; and Clevenger et al., 2003).

2. Materials and methods

Patient selection and blood sample collection

Blood sample were collected from thirty (30), women suffering from breast cancer their ages ranged from (19-60) years, and 30 blood samples collected from healthy women. All samples were subjected for molecular analysis. The blood samples that collected in EDTA tube, was stored at -20 C until used for DNA extraction. The samples were obtained from hospital Kamal Al-Sammaraee. The collection period extended from July 2023 to December 2023. Also thirty tissue samples, with breast cancer were got from Al-Khadmyaa teaching hospital. The primers that have been selected for this study to amplify portion of prolactin intron region of the gene was designed, and the details were illustrated in (table 1).

2.1. DNA extraction

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

Table 1 Sequences of primers used to amplify prolactin intron 1 region

No	Oligonucleotide	Oligosequnese	Prod.Size (bp)	GC%	Tm	Ref.
1	Forward primer	CGTAGGCTGGATTTGAAGGGT	312	52.38	54.36	NCBI
	Reverse primer	AGCGATAGATCAGGGTGCCT		55.00	53.83	
2	Forward primer	AGGGGGTAACATGCATAGCAG		52.38	54.36	NCBI
	Reverse primer	TCCCTGGATGGAGAGAGTCTG	416	57.14	56.31	
3	Forward primer	ATCCCGGGAAGTAAGCATGG		55.00	53.83	NCBI
	Reverse primer	TTGCTAGGGCTTTGGAGGTC	618	55.00	53.83	

2.2. 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 94^0C for 5 min, denaturation 94^0C for 1 min, annealing 1 min at 55^0C, extension 1 min at 72^0C 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 57^0C 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).

3. Result and discussion

3.1. Intron 1 amplification of Prolactin

samples of patients (blood, FFBE tissue) were subjected to molecular detection through PCR amplification of the PRL gene (intron 1 region) by using three specific primers predesigned for intron region of prolactin gene, the 3 primers set used in this PCR technique (PRL1, PRL2, PRL3), specific for intron 1 region of the prolactin from NCBI primer design with product length(312, 416 and 618bp respectively) which is shown in the figures (a ,b and c).

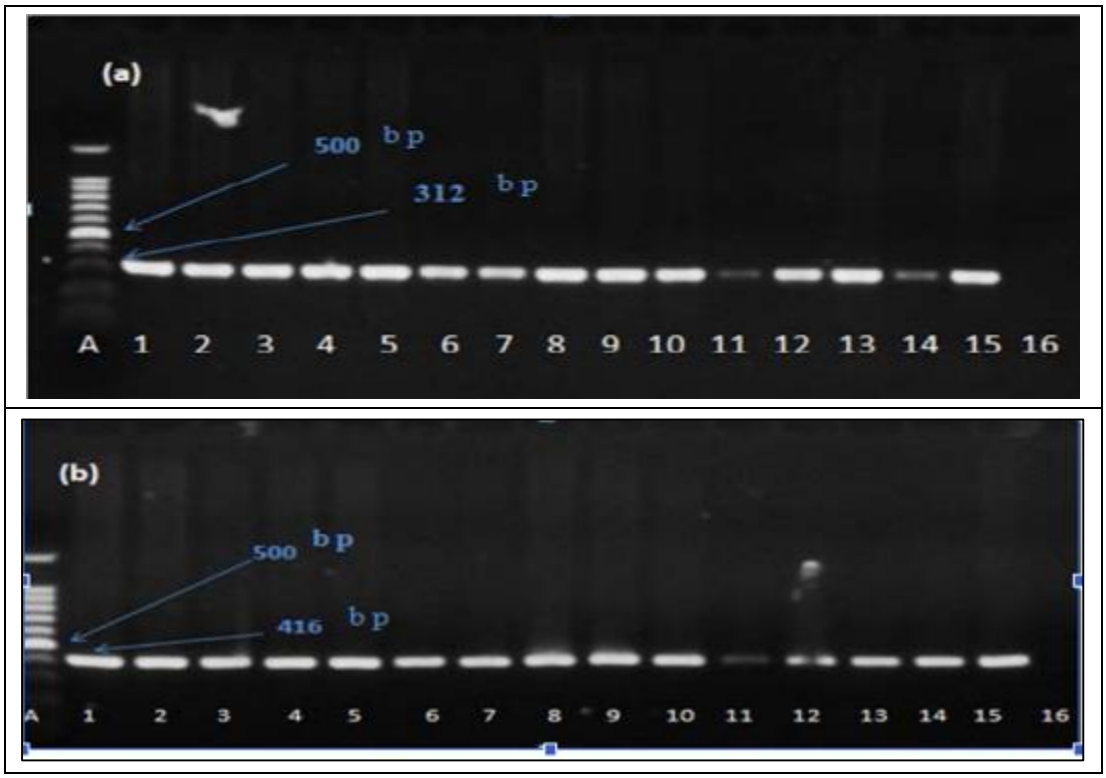


Figure 1 (a, b) Gel electrophoresis for amplification of intron 1 of PRL gene of breast cancer patients by using primer 1,(a) product size 312 bp and (b) 416 . Electrophoresis was performed on 1.5% agarose gel and run with a 80v/mAMP current for 50min.Line A=100bp ladder, line (1-6) DNA isolated from blood samples of patients, line (7-12)DNA from patient tissue, line (13-15) DNA isolated from healthy, line (16) control negative

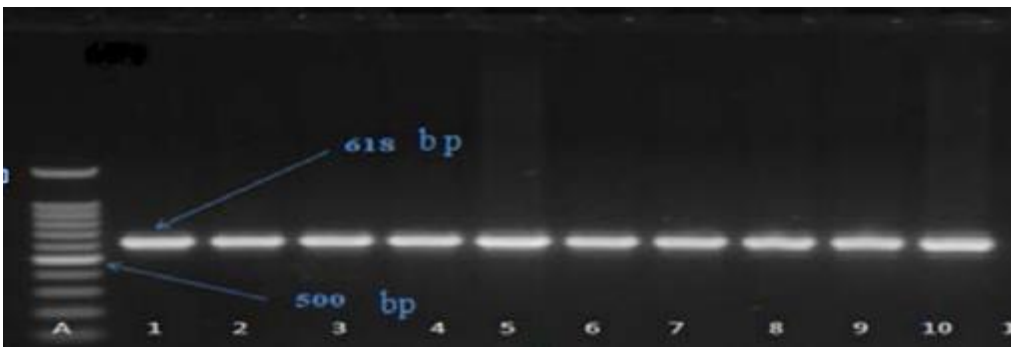


Figure 1 (c) Gel electrophoresis for amplification of PRL gene of breast cancer patients by using primer 3 which amplifies intron 1 of the gene, product size 618 bp. Electrophoresis was performed on 1.5% agarose gel and run with a

80v/mAMP current for 50min.Line A=100bp ladder, line(1-4) DNA isolated from blood samples of breast cancer patients, line(5-7)DNA from tissue, line (8-10) DNA isolated from healthy , line (11) control negative

3.2. Detection of intron 1 region of PRL gene mutations in breast cancer patients by sequencing

After amplification of genomic fragments corresponding to intron1 of the PRL gene the PCR products were (312, 618,416) shown in the above figures. By using the DNA of the above cases were selected to be sequenced in order to assess if any genetic variation in the PRL gene were known as predictors of breast cancer risk.

The sequencing was done to infected women, of breast cancer patients and for control. The results were directly matched with the Iraqi healthy, and compared to the information in the gene bank of the NCBI web site databases at www.ncbi.nlm.nih.gov using the BLAST search tool and also by using Mega 6 program. The current study utilized a forward and reverse primer for sequencing PRL gene (intron 1)of blood and tissue sample of breast cancer patients. It was found that the mutations were found around all PRL gene intron regions involved in this study, According to NCBI, this streach of intron 1 of gene, 9 mutations were detected.

The intron region of PRL region was detected and examined for the presence of any mutations or SNPs and the effect of this alteration in the function of the gene. This region was amplified using three primers, the first one amplifies the PRL at the gene region from 6169 to 6489 with product size 312bp. The second primer also amplifies the same region from 6556 to 7173, with the product size 618bp, the third primer amplified the region from 8737 to 9152, with the product size 416bp. The first and third primers were selected for gene sequencing. And it was shown that there were too many SNPs in the beginning of the gene region or in the end, so it was not considered. there were four SNPs in some samples, in breast cancer patient as shown in figure **2**

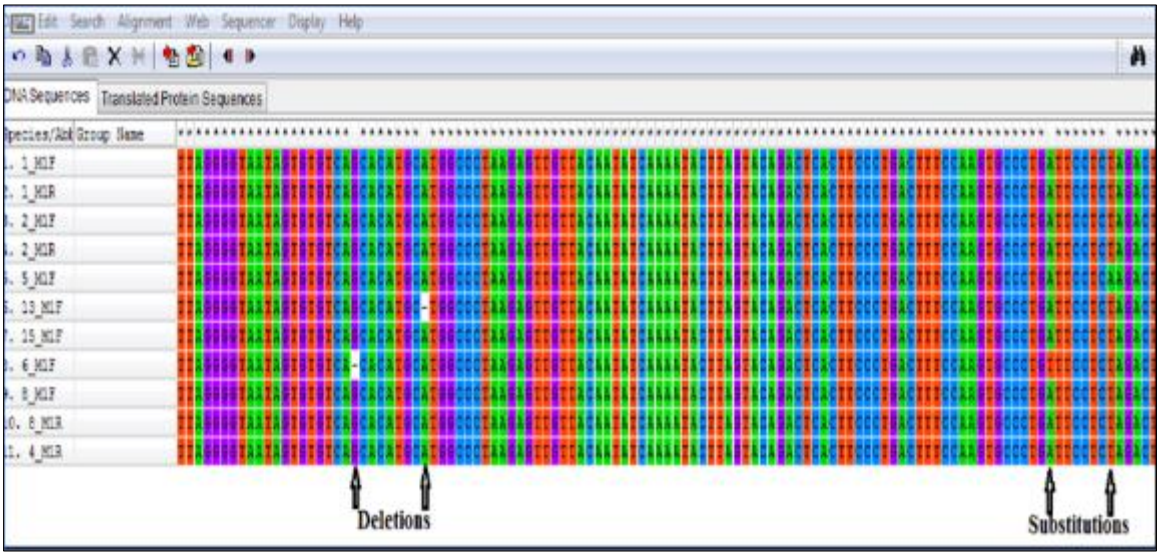
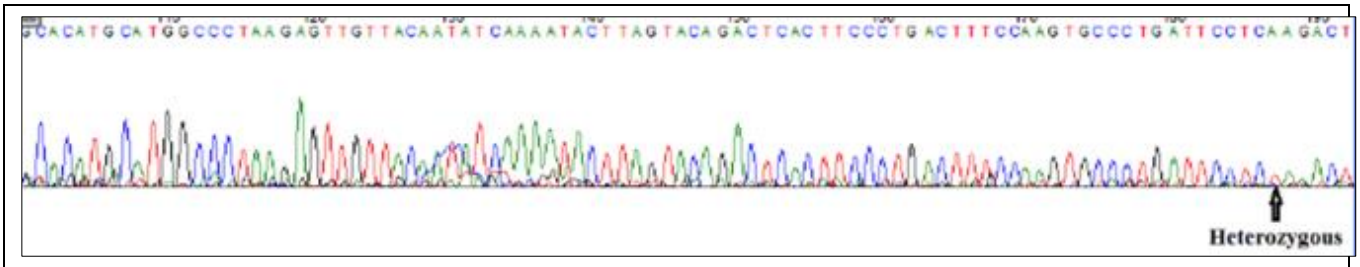
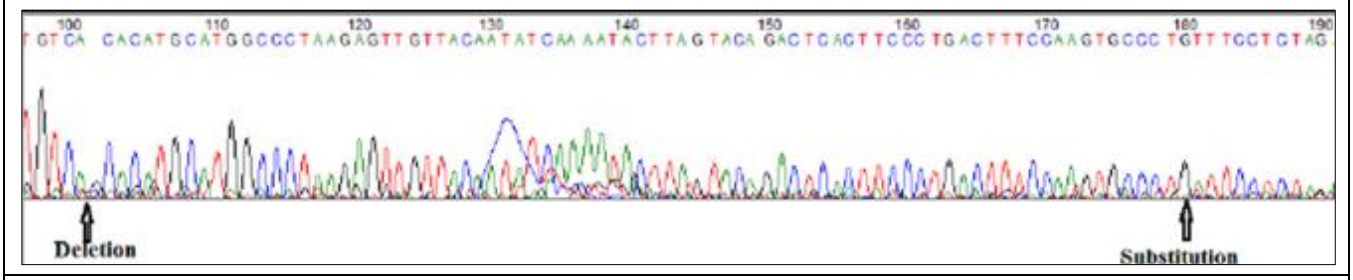


Figure 2 SNPs in intron one of hyperprolactemic and breast cancer patients by using primer 1, product size 312bp

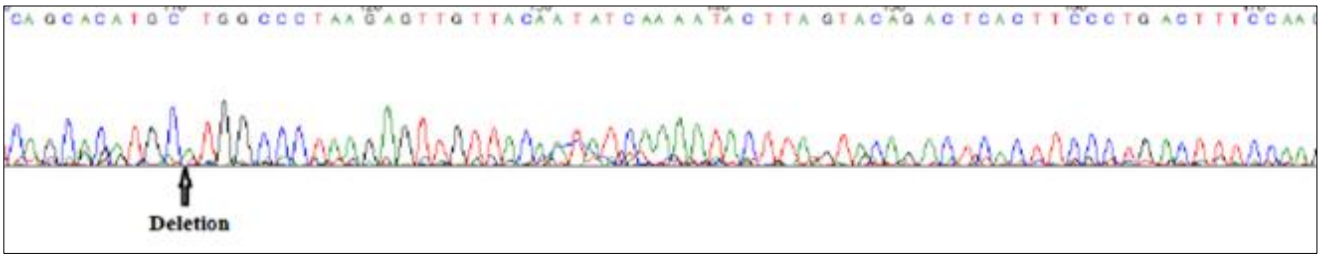
3.3. The peaks of SNPs in these samples are obvious in figure (3) a, b and c



(a)



(b)



(c)

Figure 3 Peaks of (a): breast cancer patient (Sample 5) shows the heterozygous SNP, (b): breast cancer patient (sample 6) shows the deletion and substitution mutations. (c): breast cancer (sample 13) shows the deletion mutation

The details about these mutations which appear in intron 1 in breast cancer patients when compared to NCBI are shown in figures (4) a, b and c.

Homo sapiens prolactin (PRL), RefSeqGene on chromosome 6

Sequence ID: refING_029819.1| Length: 22610 Number of Matches: 1

Range 1: 6199 to 6423 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
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Query  69    AGTGGGGGAAGTTAGGGGTAATAGTGTGTCAGCACATGCATGGCCCTAAGAGTTGTTACA  128
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             |||||||||||||||||||||||||||||||||| || | |||||
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(a)

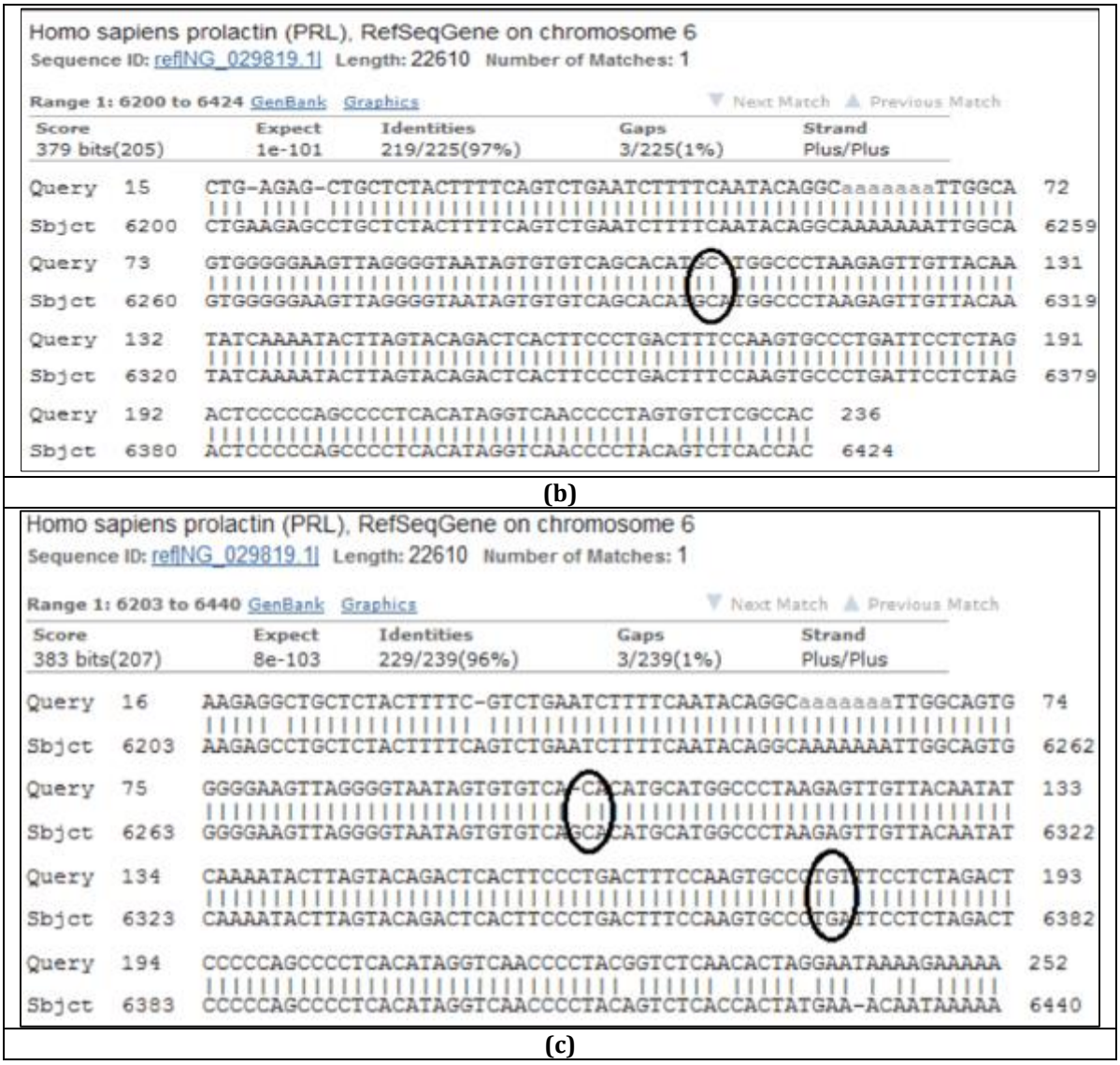


Figure 4 a, b and c: NCBI of intron1 of prolacten gene by using primer 1 (a): breast cancer patient(sample 5), b: breast cancer patient (sample 13), c: breast cancer patient (sample 6)

The three SNPs of breast cancer patients are as follows: two in sample 6 where one is substitution TGA/TGT that converts stop codon to Cys at position 147, the other is deletion mutation in which GCA/ -CA in position 69. In the same region of the gene of breast cancer patients in sample 5, there is a substitution mutation in position 156 that converts TAG/AAG that convert Stop codon to Lys. The deletion mutation is in the same region of the gene, but in other patient it is in position 77 of sample 13 that converts GCA to GC-,also in other sample but in same region which is intron 1 of the gene , there is heterozygous SNP as is clear in the peaks Fig (5).

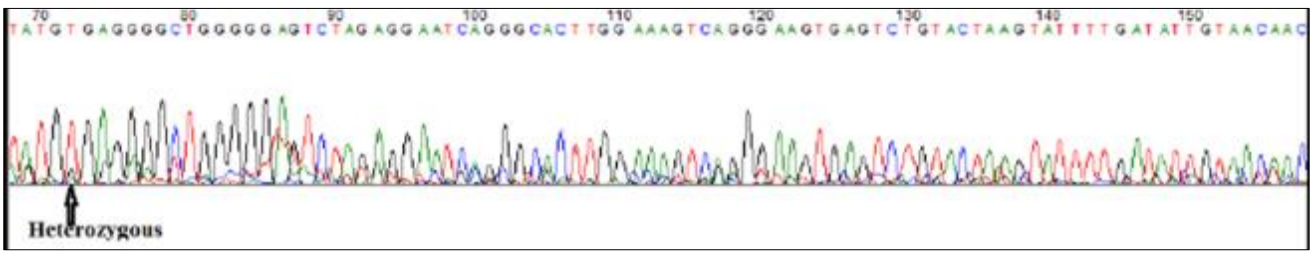


Figure 5 Peaks of intron 1 (sample 4) show the heterozygous SNP

Intron one was amplified also by using primer 3 product size 416bp and it was found that there are many substitution SNPs and also there are common SNPs between patients . In figure (6) a, the SNPs that are common between patients are clear in which AAA convert to AAT is in sample 6 and in sample 12 , in position 104 in which Lys/Asn. The other substitution mutation is in sample 6 in which GCC is converted to TGC. In the same region, but in other samples , sample 5 TAA/TAT in position 272 convert stop codon to Tyr. But in 210 position of sample 7, the ACC/GCC converts Thr/Ala. The last one is sample 9 in 277 in which TAT/TTT that converts Tyr to Phe as its clear in figure (6) a, b

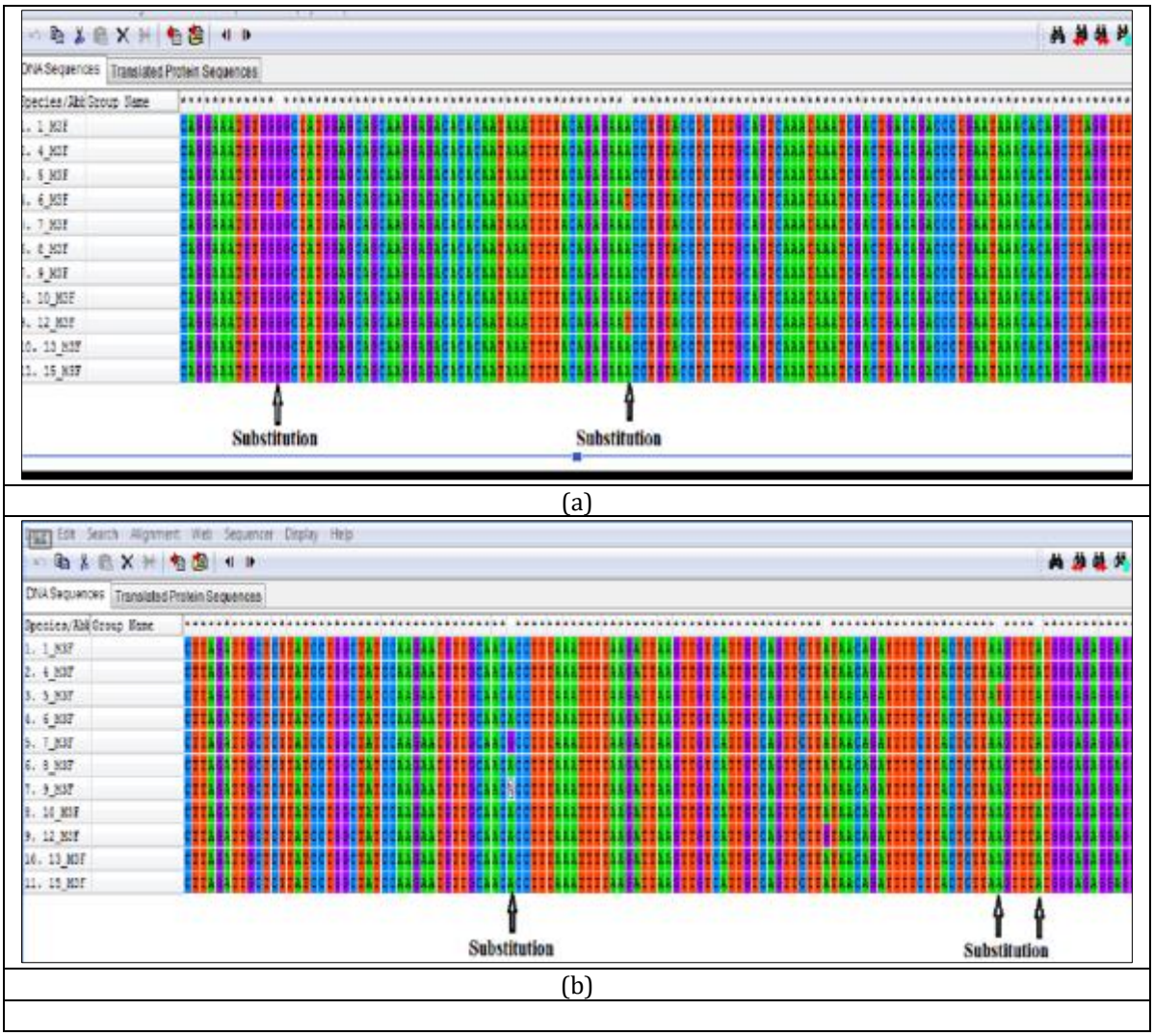
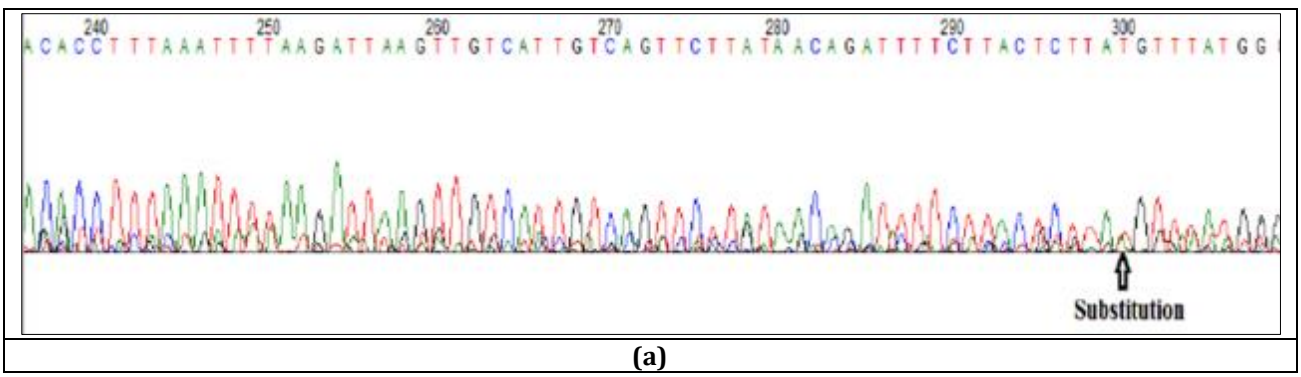


Figure 6 a and b: Substitution mutations of intron 1 by using primer 3 of breast cancer patients

The peaks for mutation samples that have mutation are clear in figure (7), a, b, c, d and e. The NCBI results of the mutations that are detected in intron one using primer (3) are obvious in figure(8), a, b, c, d, e and f.



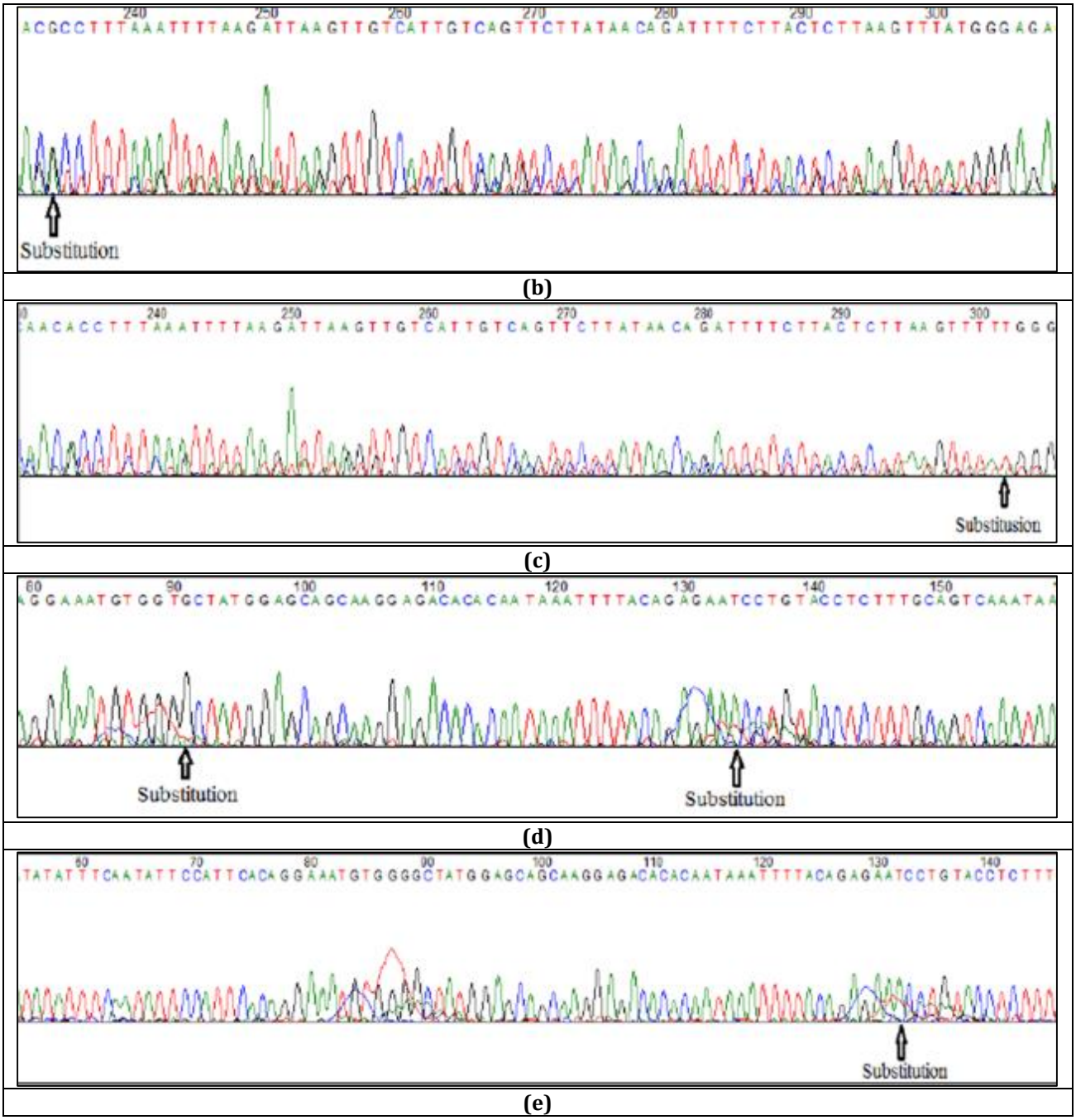
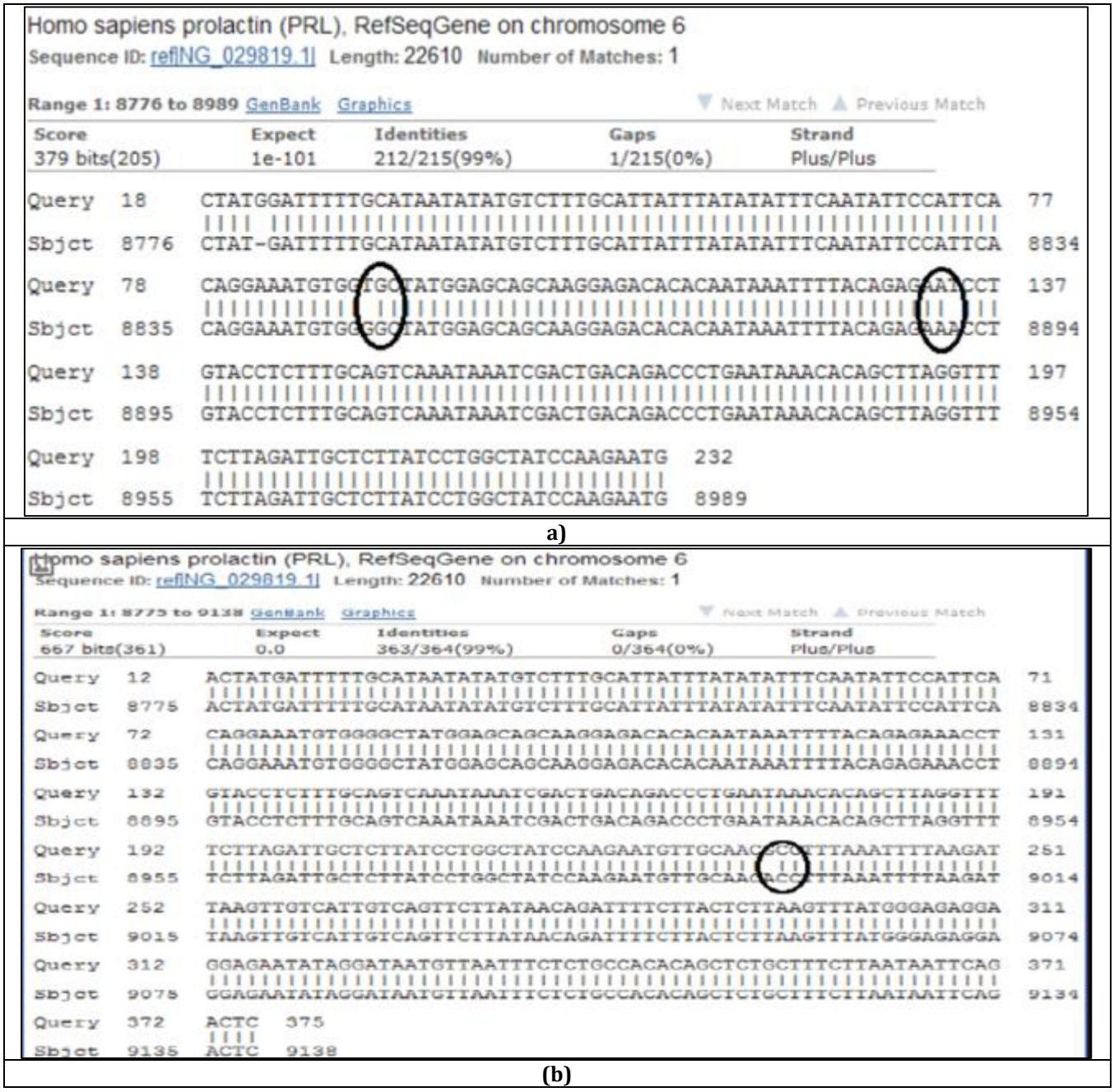


Figure 7 a, b, c, d and e: Peaks of intron 1 by using primer 3 for breast cancer patient. a, (Sample 5), b: Sample 7, c: Sample 9, d: Sample 6, e: Sample 12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Next Match Previous Match
Score Expect Identities Gaps Strand
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Sbjct 8835 CAGGAAATGTGGGGCTATGGAGCAGCAAGGAGACACACAATAAATTTTACAGAGAAACCT 8894
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Query 198 TCTTAGATTGCTCTTATCCTGGCTATCCAAGAATG 232
Sbjct 8955 TCTTAGATTGCTCTTATCCTGGCTATCCAAGAATG 8989

a)

Homo sapiens prolactin (PRL), RefSeqGene on chromosome 6
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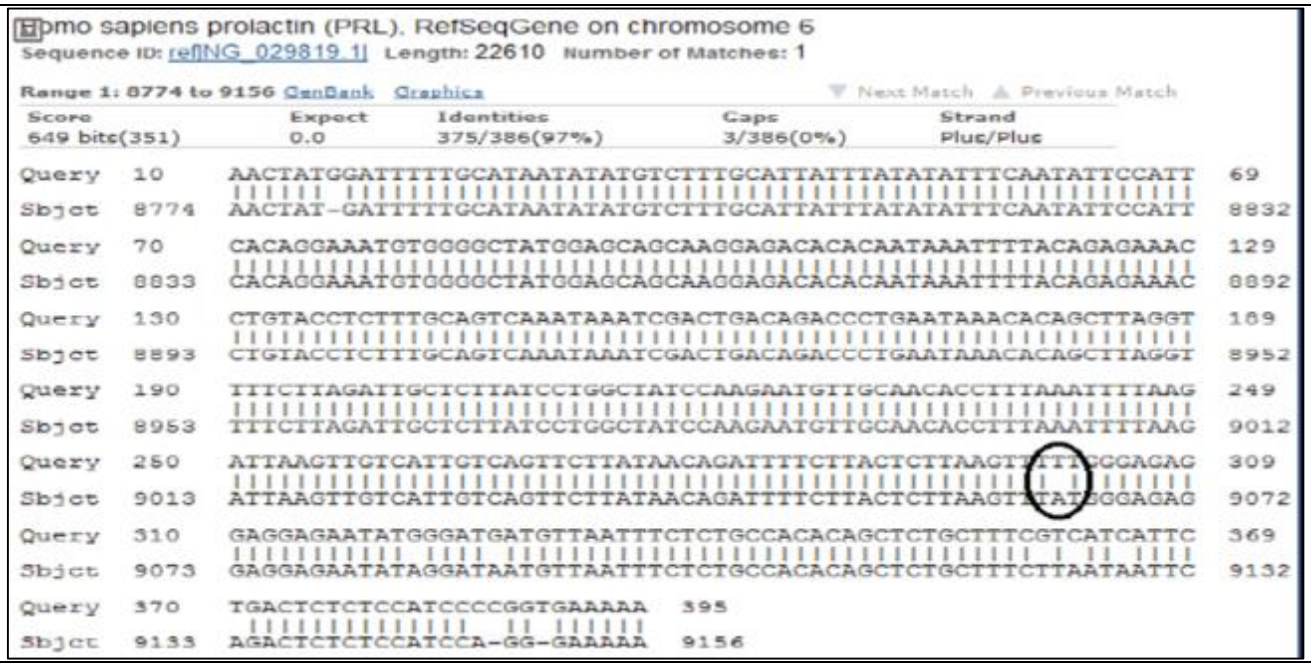
(b)


Homo sapiens prolactin (PRL), RefSeqGene on chromosome 6
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Score Expect Identities Gaps Strand
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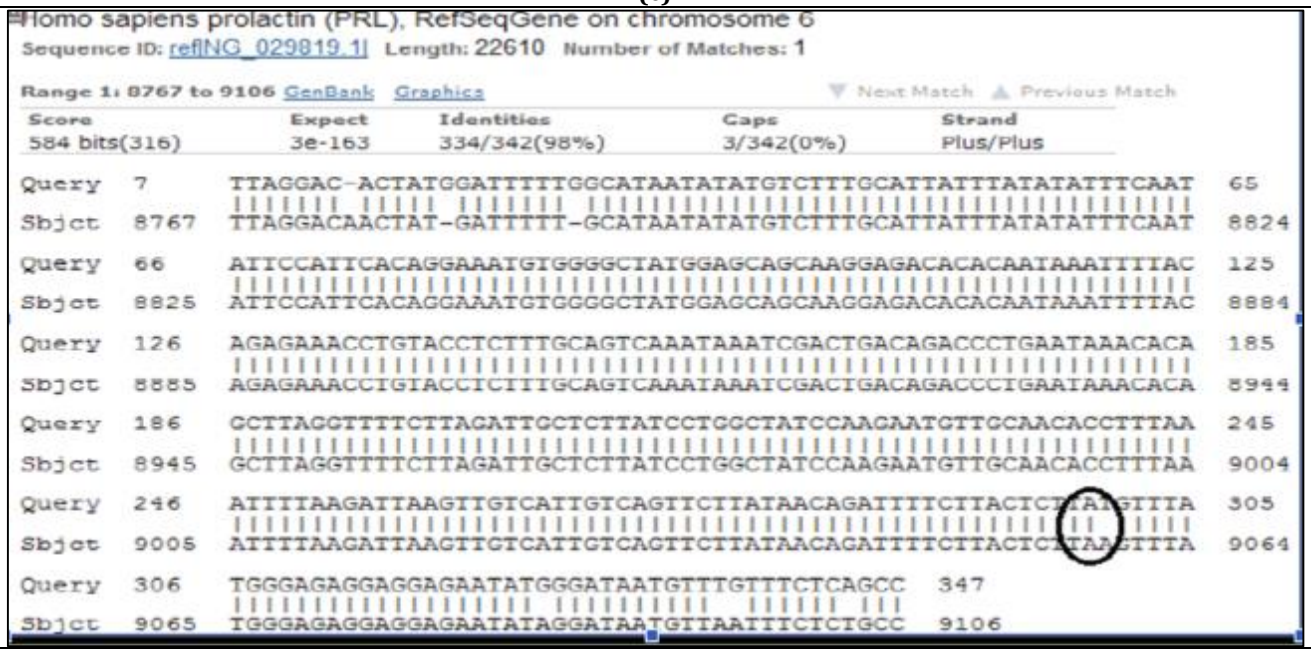
(c)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Range 1: 8767 to 9106 GenBank Graphics
Next Match Previous Match
Score Expect Identities Gaps Strand
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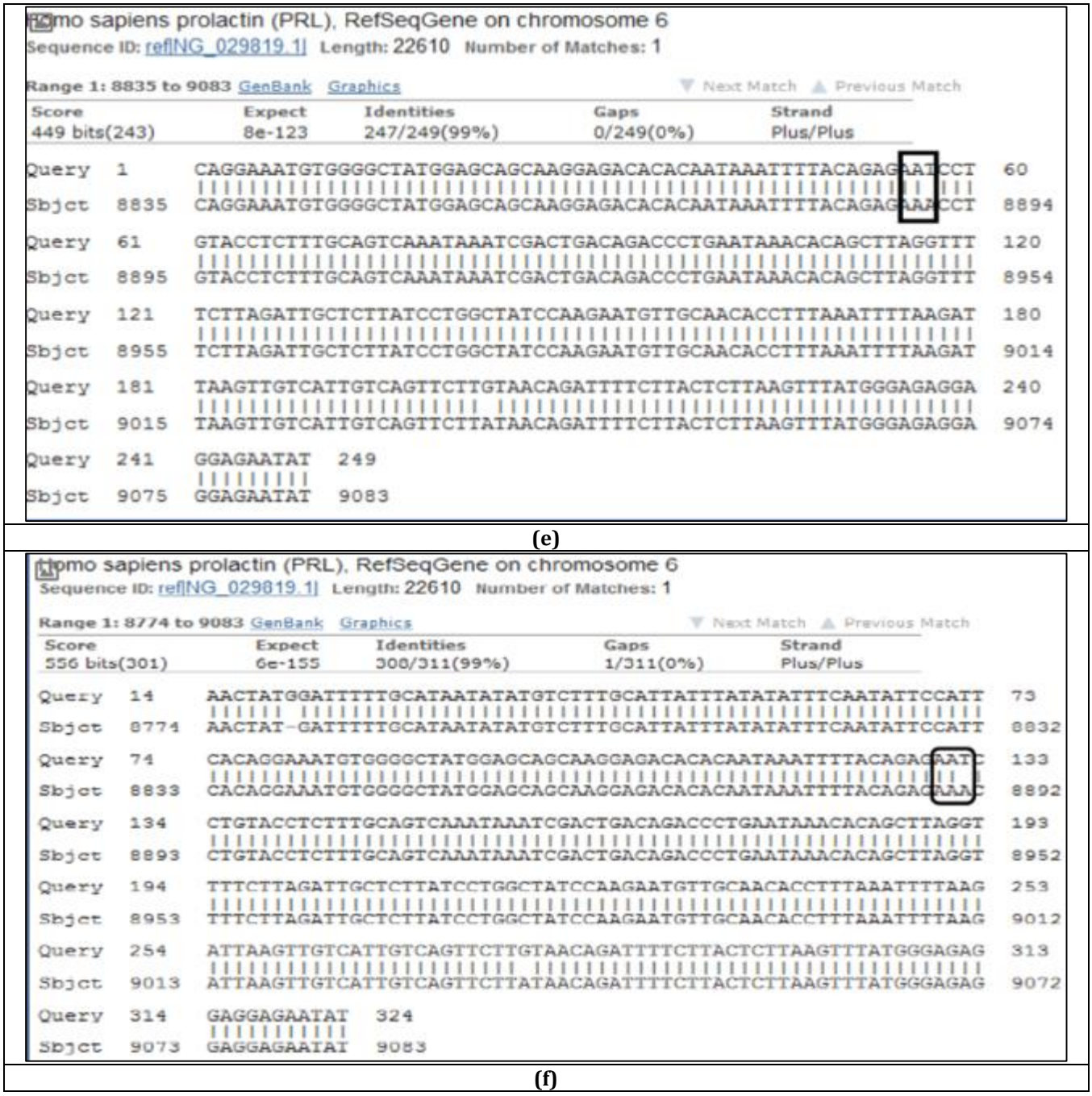

(e)

(f)

Figure 8 a, b, c, d, e and f: NCBI appear mutation of intron of PRL gene forbreast cancer patient. a:(sample 6), that appear the substitution mutations , b: NCBI of patient (sample 7). c: (Sample 9), as it appears the substitution mutation. d: patients (sample 5). e sample 12 , f: sample 6 forward

Genetic factors are important for the disease in many samples of patients, but it's not clear which region of the gene exactly contributes exactly to disease. The intron region of the PRL gene show mutations in breast cancer patients and some region of the gene shows a common mutation in some bases in some patient samples. The mutations detected in intron 1 region of PRL gene of breast cancer patients give evidence that these mutations play a part in this disease.

This result was the same in the study to Vaclavicek et al., 2006 who detected a new SNP in PRL gene showing 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. It was significantly associated with an increased breast cancer risk.(Vaclavicek et al., 2006). Besides, a rare homozygous genotypes of the (A/T) SNP near exon 2 and the (G/A) SNP near exon 5 were 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 this study a two heterozygous SNPs were detected (Stop/Trp) in two breast cancer samples. From this study, it was shown that here is mutation in PRL gene and some mutations are common with breast cancer patients. So this association was detected between the PRL gene mutation and breast cancer risk. This has been 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 this study, there are SNPs in breast cancer patients and the same in exon 2 and exon 4. This may be PRL is involved in mammary gland growth and differentiation, so overexpressing of prolactin or variation happens in prolactin gene, patients will increased mammary tumorigenesis (Ben-Jonathan et al., 2002). This agrees with a study by Faupel-Badger et al., (2010), who reported that higher prolactin levels were associated with an increased breast cancer risk.

It is also agrees with Lee et al., 2007, 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. Hormone PRL physiologically influences the mammary gland in several ways during development, growth and stimulation of milk protein gene transcription (Wennbo et al., 1997).

Besides PRL is important 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 cases of tumor growth. The role of it on breast cell proliferation is that the tumor growth promots effects of PRL signaling in the mammary gland which are well documented in animal models (Nevill et al., 2002; Ormandy et al., 2003).

This result also agrees with Nitze et al., (2013), who found that as prolactin has been implicated in tumorgenesis, it is important for proliferation and differentiation of the breast epithelium. It is shown that PRL and receptor are co-expressed in breast cancer tissues and cell lines and thus PRL has been suggested to promote the growth of the carcinomas in autocrine/paracrine fashion (Ben-Jonthan ,2002).

The mechanisms that have been suggested to explain the 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(Vyas, 2012).

This agrees with the study of Plutnikov (2009) who shows that as the PRL signaling is mediated by its similar 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 of PRLR turnover results in augmented PRL signaling and PRL induced transcription.

In the present study, it was found that there are many mutations in intron region of the gene as its clear in the above figures conserving the mutations in intron 1. This agree with the result obtained by the Iraqi study which reported the mutations in intron 1 and 2 of prolactin gene of infertile hyperprolactinemic women.

Nore et al. (2013) reported the mutations in hyperprolactinemic patients in intron region of the PRL gene, and thus they considered them as genetic markers for breast cancer Moreover, it was found that there are 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 and breast cancer clear. Thus, it agrees with the (Vaclavicek et al., 2006), who showed a significant association between the promoter SNPs of the prolactin gene and breast cancer. positive results for the association of PRL gene polymorphism in breast cancer patients.

Direct Actions on Mammary Epithelia Extensive studies of the direct actions of PRL on breast cancer cells *in vitro* have demonstrated increased proliferation and cell turnover (Marano et al 2014)

Various studies have demonstrated the proliferative, anti-apoptotic, and angiogenic effects of PRL in human breast epithelium, also pointing to a presumptive pro-carcinogenic action(Arendt et al 2011), (Clevenger et al 2003)

Also many studies showed that , genetic variation in human PRL gene has also been associated with breast cancer risk, specific clinicopathological features, and clinical outcome of the disease(Booms et al 2019),(Ellingjord-Dale et al).. (López-Ozuna et al 2016).(Mohr et al 2016)

4. Conclusion

In the present study it was found that, there were many mutations , SNPs were detected in intron 1 region of prolactin gene of blood and tissue samples collected from blood and tissue of breast cancer patients in compared with healthy samples .The positive results for the association of polymorphism in PRL intron 1 region of the gene with the breast cancer disease confirmed as the same SNP were found in the same position of many patients. As shown a significant association were detected between SNPs of the prolactin gene and breast cancer thus the mutation in intron region of the PRL gene, can be considered as genetic marker for the infection with breast cancer .

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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