

Sequence and Structure Analysis of CRP of Lung and Breast Cancer Using Bioinformatics Tools and Techniques

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In the present study, bioinformatics approach has been adopted to explore the sequences and structures analysis of CRP of lung and breast cancer and compares with normal sequence from NCBI. The present study was aimed to investigate the possibility of using CRP as a marker for patients with lung and breast cancer. Also the effect of mutation on the physicochemical properties and structure of CRP. 40 blood and serum samples were examined from patients with lung and breast cancer (aged between 23 and 45 years old). Qualitative test was done to detect the presence of CRP in the patient's serum. The qualitative test showed that 3(7.5%) patients give positive result and 37(92.5%) patients with lung and breast cancer give negative result to presence of CRP in the serum. In patient with lung cancer five missense mutations and four deletion mutations detected. While in the patient with lung cancer four missense mutations, six deletion mutations and eight insertion mutation detected by BLAST. One point mutation appeared in all patients at same site and has same effect, this meaning there are relationship between this mutation and cancer disease. This mutation recorded in NCBI, DDBJ and ENA with the numbers LC276938 and LC276937. The present study determined the physico-chemical properties of CRP such as their hydrophilic nature; alpha-helical structure and 3D structure. The results of present study show that CRP consider non-specific marker for patient with Lung and breast cancer. Also the mutations on CRP gene effected on the structure and physico-chemical properties of C-reactive protein.

Keywords: CRP, Bioinformatics tools, Breast cancer.

C-reactive protein was one of the most important proteins in the medical field and in identifying disease states associated with inflammation. C-reactive protein (CRP), is the one of the most important protein, belonging to pentraxin family of proteins¹. The Cytogenetic location for CRP gene: 1q23.2 which is the long (q) arm of human chromosome 1 at position 23.2, this position contains gene which encode to CRP². C-reactive protein produced in the liver in response

to IL-6³. Interleukin-6 consider one of tumour necrosis factor⁴. CRP has been associated with cancer size and the stage of disease, and also can it be used as a "cancer marker" in the determining of disease activity⁵. Chronic inflammation may be a caused factor in a variety of cancers. In general, the exposure to inflammation for long time, lead to the risk of cancer. The relationship between inflammation and cancer was that central role in the regulation of inflammatory and

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immune response was IL-6. And also IL-6 plays important roles in cancer progression related to proliferation, migration, and angiogenesis⁶. The national cancer institute consider CRP as marker for cancer disease⁷. Cancer considers one of the most important health problems of the current era and also a leading cause of death among population. Cancer can simply be defined as a malignant tumor or malignant neoplasm, It includes a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. It can also be defined as a group of disorders that are characterized by uncontrolled division of cells and the ability of these abnormal cells to spread⁸. The most frequent cancer among women was the breast cancer. It's the female malignant neoplasia with the highest incidence in the world⁹. Breast cancer remains a major problem of women's health and its incidence is increasing in developing countries¹⁰. Another important type of the cancer was the lung cancer. The lung cancer considered the most common cancer in terms of both incidence and mortality. Also it consider the second most commonly occurring of cancer in the world and it is leading to the cancer-related cause of death¹¹. The aims of this research paper is to investigate the possibility of using CRP as a marker for cancer, computational analysis of CRP using bioinformatics methods, sequence and structural analysis of CRP of breast and lung cancer using bioinformatics methods.

MATERIALS AND METHODS

Blood Sample collection

The sample size of this research paper is 40 human patients with breast and lung cancer. For each patient drawn three milliliter of whole blood for patients was obtained under aseptic conditions from each subject by a vein puncture using a disposable syringe. Whole blood was divided into two parts: first part (about two milliliters) was collected in sterile EDTA tube for genetic test. The second part of blood collected in gel tube were separated by centrifugation at 3000 rpm for 10 minutes for qualitative test. The blood and serum samples were subjected to freezing at -20°C ¹².

Qualitative test

The serum samples containing human CRP were obtained from patients with lung and

breast cancer, that give positive and negative results for qualitative test by using protocol in the Latex kit for C-Reactive protein (Cat. No.: NS 514001/ Salucea company).

DNA extraction from blood samples

The human DNA extraction from the whole blood of the human patients by using protocol in the gSYNCTM DNA Extraction Kit (Cat. No.: GS100/Genaid company).

Estimation of DNA concentration and purity

The human DNA concentration and purity of the samples extracted from whole blood were estimated by using nanodrop (Acta gen/USA) by putting one microliter of the extracted DNA was add to the nanodrop machine to evaluate the concentration in $\text{ng}/\mu\text{l}$. The purity (1.6-1.8) means DNA high purity [13].

Agarose Gel Electrophoresis

After genomic DNA extraction from blood, agarose gel electrophoresis was adopted to confirm the presence and integrity of extracted DNA¹³. The samples were carefully loaded into the individual wells of the gel. Then the electrical power was turned on at 56 volt for 30 minutes. Afterwards, the DNA moved from the cathode(-) to the anode(+) poles. The Ethidium Bromide stained bands in the gel were visualized using UV transilluminator at 365 nm and photographed.

Primer design for CRP gene

The full CRP gene sequences and annotation taken from Genome Database of the national center for biotechnology information (NCBI), the NCBI reference sequence: NC_000001.11. Using primer3 software (<http://bioinfo.ul.ie/primer3>) for the three Exon of CRP design^{14,15}, table (1) shows the sequences of the designed for the forward and reverse primers. Then the primers provided by Bioneer Company /Korea in a lyophilized form and using the primers in PCR.

PCR

The reaction mix was constituted by $30\mu\text{M}$ KCl, $10\mu\text{M}$ Tris-HCl (PH 9.0), $1.5\mu\text{M}$ MgCl₂, $250\mu\text{M}$ each dNTP, $2\mu\text{l}$ each primer, 1U of Taq DNA polymerase, $5\frac{1}{4}\text{L}$ of the DNA template (sampl) and ultrapure deionized sterile distilled water up to $20\mu\text{L}$. The thermocycler conditions consisted of the program was set in the thermo cycler to amplify the target DNA for CRP1, CRP2 and CRP3 primers in table(2):

Electrophoresis (1 h at 75 V) was

performed with 5 μ L of the reaction solution in a 2% agarose gel with ethidium bromide. PCR products were visualized under UV light and photographed. The DNA ladder (100pb) was used to estimate the molecular size of the bands.

DNA sequencing

The result of PCR (Polymerase Chain Reaction) for patient with breast and lung cancer whom gave clear band after PCR analysis of the analyzed (CRP) gene, sent to macrogen company/ Korea, for sequencing the three Exon of CRP gene for each samples.

Compared between the DNA sequence of CRP gene for samples and CRP gene retrieved from NCBI

using BLAST server (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>)¹⁶, for detection the location of mutation in CRP for patients.

Translation the DNA sequence of CRP gene to amino acids sequence

using BLASTX server (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) for translation DNA nucleotide to amino acids¹⁶.

Physicochemical properties Prediction

using online tool Prot Param (<http://us.expasy.org/tools/protparam.html>) for Physico-

chemical properties of the CRP for the patients with lung and breast cancer, also the CRP retrieved from NCBI. The parameters computed by Prot Param include the molecular weight, theoretical pI, amino acid composition, atomic composition, instability index, aliphatic index and grand average of hydropathicity (GRAVY)¹⁷.

Protein Structure Prediction

Using PSIPred online tool (<http://bioinf.cs.ucl.ac.uk/psipred/psiform.html>) for Secondary structure has been predicting^{18,19} where the FASTA format of the sequence was given as input. It provides the structural information of the protein sequence in form of coils, helices and using PHYRE2 software (<http://www.sbg.bio.ic.ac.uk/phyre2/html>) for 3D-structure prediction²⁰. The modeling involves four basic steps, first searching structure showing homology with target, then selecting a best template having maximum identity with the target sequence which follows its alignment with the target and modeling the structure. On the other hand using swiss-model (<http://swissmodel.expasy.org/>) an automated system for modeling the quaternary structure of a protein from its amino acid sequence using homology modeling techniques²¹

Table 1. Names and sequences of the primers used for CRP gene amplification

No.	Primer name	Type	Sequence	Size product
1	CRP1	Forward	5'-ATC CAG GCA GGA GGA GGT AG-3'	238pb
		Reverse	5'-CCC CCA TAC CTC AGA TCA AA-3'	
2	CRP2	Forward	5'-TCG AGG AAG GCT TTT GTG TT-3'	597pb
		Reverse	5'-CTG GGG TTT GGT GAA CAC TT-3'	
3	CRP3	Forward	5'-TGT TTG CTT GCA GTG CTT TC-3'	485pb
		Reverse	5'-TGG GCA GTC CAG GTG TAG AT-3'	

Table 2. The program was used for amplification the target DNA for (CRP1, CRP2 and CRP3 primers).

No.	Type of steps	Temperature	Time period	No. of cycles
1	Pre-denaturation	94°C ⁽¹⁾	5 minutes	1
2	Denaturation	94°C ⁽¹⁾	30 second	30
3	Annealing	58°C ⁽²⁾ , 57°C ⁽³⁾	45 second	
4	Extension	72°C ⁽¹⁾	1 minute	
5	Final Extension	72°C ⁽¹⁾	5 minutes	1
6	Storage	4°C ⁽¹⁾	"	

¹Constant for the three primers, ²The annealing step for CRP1 and CRP2 primers.

³The annealing step for CRP3 primer

RESULTS AND DISCUSSIONS

Serum specimens were tested for presence of CRP using latex kit for CRP. However, the results obtained from qualitative test were 1(3.3%) patient give positive result and 29 (96.7%) patients with breast cancer give negative result to CRP, while the study of²², Which proved that the CRP was act as marker for predictor and to identify the

risk of breast cancer. About 2 (20%) patients with lung cancer give positive result and 8(80%) patients give negative result for CRP, while study of²³, that proposed the levels of CRP in serum sample are increased in patients with lung cancer. All these results agree with study of²⁴, that proved CRP was non-specific marker for inflammation. Genomic DNA from the blood samples was extracted following the standard protocol which used in

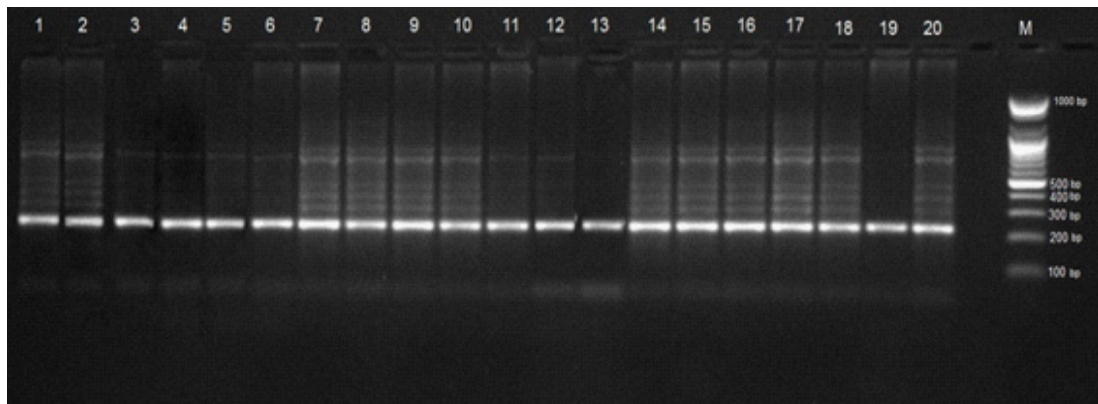


Fig. 1. Detection of PCR product DNA bands of CRP gene with use of CRP1F CRP1R primers (238 bp.). The amplified fragment were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide at 60 volt for 90 minute. Photographed under UV light.

1. Lanes (1-10) patient with breast cancer gave band for amplified fragment.
2. Lanes (11-20) patients with lung cancer gave band for amplified fragment.
3. M:Marker DNA ladder size (100bp)

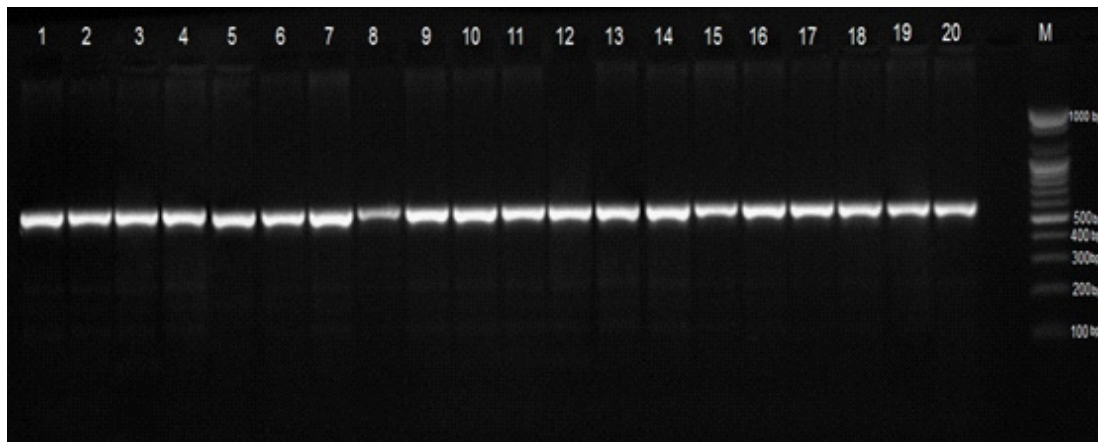


Fig. 2. Detection of PCR product DNA bands of CRP gene with use of CRP2F and CRP2R primers (597 bp.) . The amplified fragment were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide at 60 volt for 90 minute. Photographed under UV light.

- 1.Lanes (1-10) patient with breast cancer gave band for amplified fragment.
2. Lanes (11-20) patients with lung cancer gave band for amplified fragment.
3. M: Marker DNA ladder size (100bp)

many researches and genetic studies according to¹³. The quantification of DNA measured by Nanodrop had revealed that the DNA concentration ranged(3-5) ng/μl and purity ranged was (1.6-1.8). The result demonstrated that the purity of the extracted DNA in all samples were sufficiently high for PCR analysis .

PCR

The present study used PCR technique for DNA isolated from the blood from patients to amplify the 3 exons of the CRP gene. By using three primers(CRP1,CRP2 and CRP3 primer),that were designe by primer3 software and obtained from

Bioneer Company (Korea). The PCR results were interpreted by the presence or absence of specific bands of amplified gene on 2% agarose. In this study, three primers were screened for PCR analysis using 40 DNA samples that were extraction from the whole blood of patients. The results of using first set of primers (CRP1F and CRP1R), second set of primers (CRP2F and CRP2R) and using third set of primers (CRP3F and CRP3RR) showed amplified fragment (238pb,597bp and485bp. respectively) a clear band by electrophoresis on a 2% agarose gel at 60 volt for 90 minute, of 40 DNA samples as shown in figure(1).

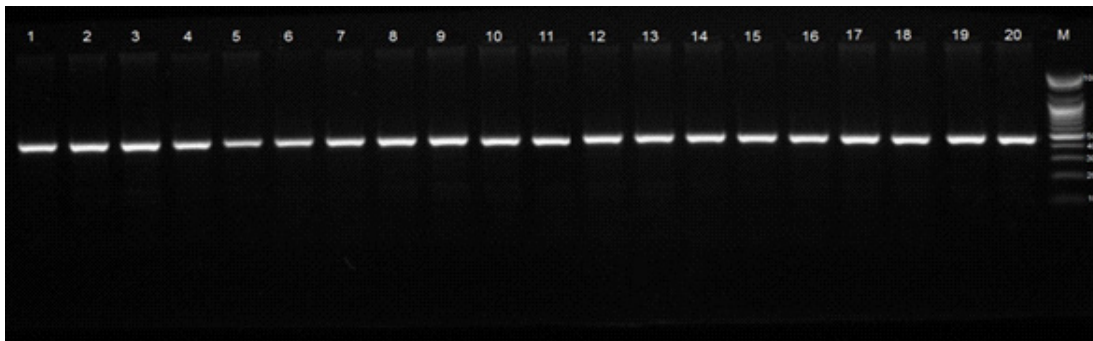


Fig. 3. Detection of PCR product DNA bands of CRP gene with use of CRP3Fand CRP3R primers (485 bp.). The amplified fragment were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide at 60 volt for 90 minute. Photographed under UV light.
 1. Lanes (1-10) patient with breast cancer gave band for amplified fragment.
 2. Lanes (11-20) patients with lung cancer gave band for amplified fragment.
 3. M: Marker DNA ladder size (100bp)

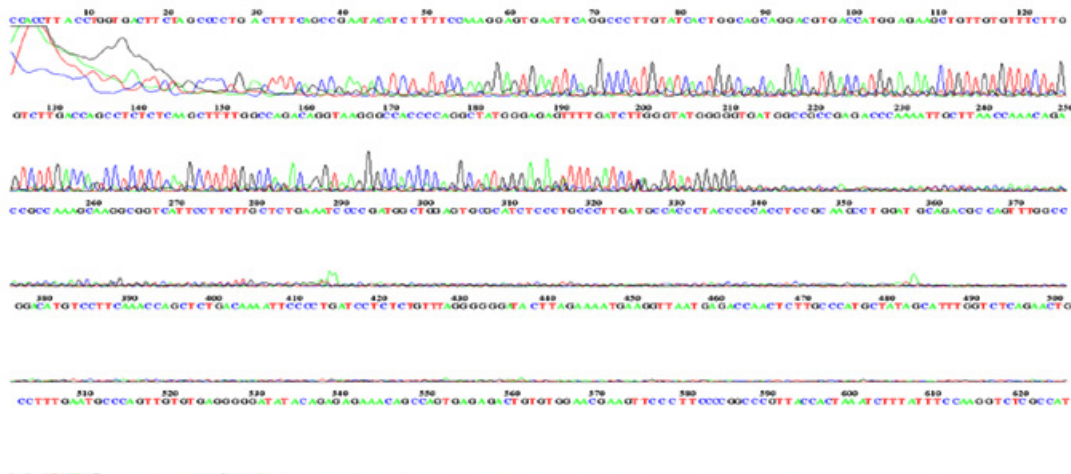


Fig. 4. The chromatogram for the forward nucleotide sequence of amplified the first primer for patient with lung cancer by DNA sequencer

The result of using second primer (CRP2F and CRP2R) showed amplified fragment (597bp.) as a clear band by electrophoresis on a 2% agarose gel at 60 volt for 90 minute, as shown in figure (2) in all patients and control.

The result of using third set of primer (CRP3F and CRP3RR) showed amplified fragment (485bp.) as a clear band by electrophoresis on a 2% agarose gel at 60 volt for 90 minute, as shown in figure (3) in all patients and control.

The outcomes of the CRP gene amplification using PCR analysis represented that all patients gave positive result (formation bands). This results showed that the product of amplified first set of primers (CRP1F and CRP1R), second set of primers (CRP2F and CRP2R) and third set of primers (CRP3F and CRP3RR) of CRP gene find in all patients.

Sequencing of C-reactive protein gene

The current study utilized forward primers for two patients by direct sequencing.: first with lung cancer(4) and second patient with breast cancer figure(5).

The comparison between DNA subjects and reference sequence are shown in figure (6)

Genotype analysis of CRP gene for the PCR product of the primer indicated of much genetic alteration. The effect of mutations in translation and the result revealed that there were five missense mutations and four deletion mutations in patient with breast cancer. The four missense mutations ,six deletion mutations and

eight insertion mutation in the patient with lung cancer table(3).

Finally the thymine transversion by adenine at the site 159714441 in the first Exon of CRP gene on the long arm of first chromosome. This mutation recorded in NCBI ,DDBJ and ENA with the numbers LC276937 and LC276938 this point mutation effected on the translation and cause missense mutation , when methionine replaced by lysine appeared in all patients at same site and has same effect, this meaning there are relationship between this mutation and cancer disease. And also this mutation detection in multiple tumor samples, so it can be consider the cause of cancer disease these result agree with study²⁵ demonstrated that Tay–Sachs disease is caused by a genetic mutation in the HEXA gene on chromosome 15. these result agree with study²⁶ demonstrated that sickle-cell anemia is caused by a point mutation in the ²-globin chain of hemoglobin, caused replace glutamic acid with amino acid valine at the sixth position. these result agree with studies of^{27,28} demonstrated that neurofibromatosis is caused by point mutations in the neurofibromin gene and these result agree with study²⁹ demonstrated that cystic fibrosis is caused by a mutation in the CFTR gene.

Primary structure of C-reactive protein

The sequence of CRP for patients with lung and breast cancer consist from 219 and 211 amino acids respectively. While the sequence of CRP retrieved from NCBI consist from 224 amino acids.

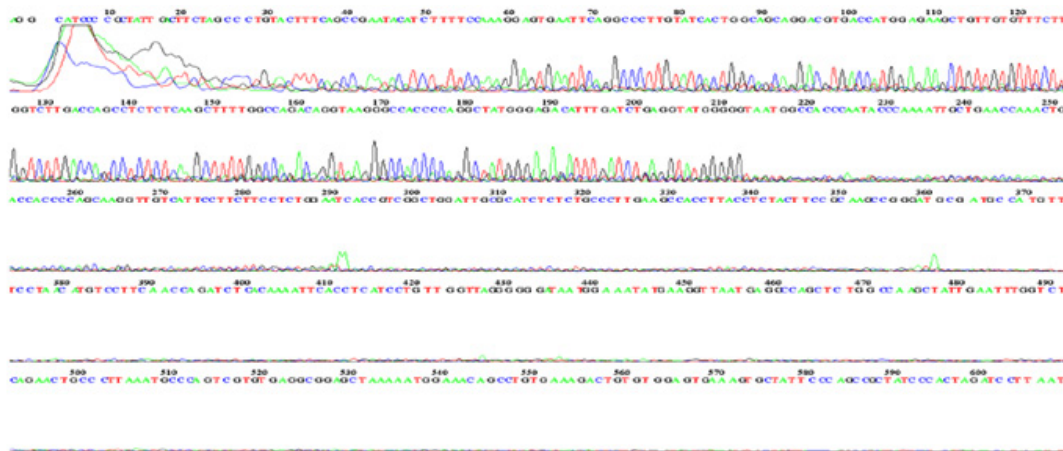


Fig. 5. The chromatogram for the forward nucleotide sequence of amplified the first primer for patient with breast cancer by DNA sequencer

Sequence analysis

Primary structure analysis provided the physicochemical properties of CRP. Molecular weight of CRP for the patients with lung and breast cancer was (24338.88 and 23508.82 MW). While the molecular weight for CRP that retrieved from NCBI FASTA format-protein was (25038.58MW). Most abundant amino acid was Ser(S) in CRP for lung and breast cancer 9.6% and 9.5% respectively, while in CRP that retrieved from NCBI was 10.3%, Gly(G) 8.7% and 8.5% respectively, while in CRP that retrieved from NCBI was 8.0%, Leu(L)

8.7% and 8.5% respectively, while in CRP that retrieved from NCBI was 8.9%, val (V) 8.2% and 8.5% respectively, while in CRP that retrieved from NCBI was 8.5%. A protein whose instability index is smaller than 40 is predicted as stable, a value above 40 predicts that the protein may be unstable[20]. Prot Param server predicted that CRP for patients with lung and breast cancer were stable. And also the CRP that retrieved from NCBI was stable. The isoelectric point of a protein is an important property, because it is at this point that the protein is least soluble²⁰. Computed

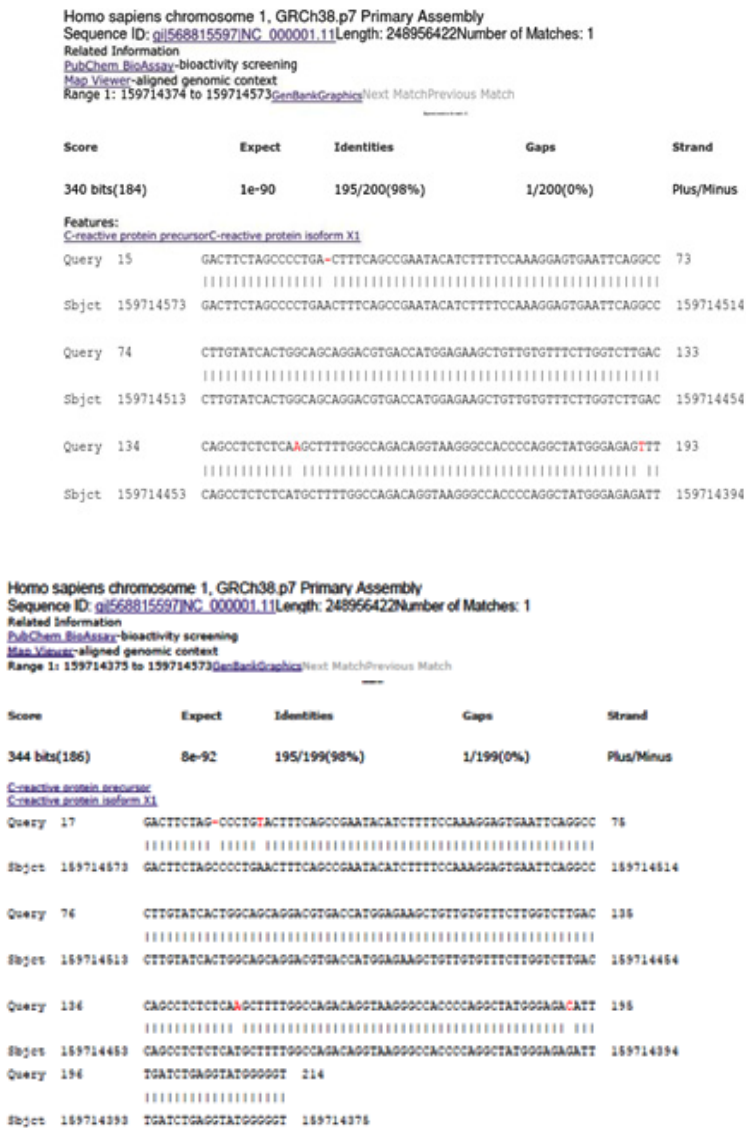


Fig. 6. Shown simple part of the comparison results between DNA subjects and reference sequence by BLAST

Table 3. Types of mutations for the Exon of CRP gene sequencing

Types of disease	No.Of primer	Wild genetic code of DNA	Mutations in genetic code of DNA	Type of mutations at DNA level	Wild genetic code of RNA	Mutations in genetic code of RNA	Effect of translation	Site of mutations
Breast cancer	First	GAA	GTA	Transversion	GAAGlutamate	GUAValine	Missense mutation	159714558
Breast cancer	First	GCC	G - C	Deletion mutation	GCCAlanine	G - C	Frame shiftMutation	159714564
Breast cancer	First	AGA	ACA	Transversion	AGAArginine	ACAThreonine	Missense mutation	159714397
Breast cancer	First	ATG	AAG	Transversion	AUGMethionine (start code)	AAGLysine	Missense mutation	159714441
Breast cancer	Second	AAG	A - G	Deletion mutation	AAGLysine	A - G	Frame shift mutation	159714077
Breast cancer	Second	CCA	CAA	Transversion	CCAProline	CAAGlutamine	Missense mutation	159713546
Breast cancer	Third	AAA	A - -	Deletion mutation	AAALysine	A - -	Frame shift mutation	159712764
Breast cancer	Third	TGT	TAT	Transition	UGUCysteine	UAUTyrosine	Missense mutation	(-)159712763(-)
Breast cancer	Third	CTG	C - G	Deletion mutation	CUGLeucine	C - G	Missense mutation	159712443
Lung cancer	First	GCC	G-C	Deletion mutation	GCCAlanine	G-C	Frame shift mutation	159712333
Lung cancer	First	GAA	GTA	Transversion	GAAGlutamate	GUAValine	Frame shift mutation	159714564
Lung cancer	First	AGA	ACA	Transversion	AGAArginine	ACAThreonine	Missense mutant	159714558
Lung cancer	First	ATG	AAG	Transversion	AUGMethionin (start code)	AAGLysine	Missense mutant	159714397
Lung cancer	First	GAG	GGG	Transition	GAGGlutamate	GGGGlycine	Missense mutant	159714386
Lung cancer	Second	TCA	T -	Deletion mutations	UCASerine	U -	Frame shift mutation	1597140561
								59714055

Mutation

Genetic code for amino acid produces from insertion mutation

isoelectric point of CRP for patients with lung and breast cancer were 5.75 and 5.26 respectively also the PI for CRP that retrieved from NCBI was 5.76 below 7 so they are likely to precipitate in

acidic buffers. This result agree with studies of^{30,31} demonstrated that the mutation cause large changes in the sequences this effected on physiochemical properties of protein especially on protein stability.



Fig. 7. Secondary structure of CRP for patient with lung cancer(A), breast cancer(B) and CRP that retrieved from NCBI(C) respectively by PSIPred

Structure analysis

The secondary structure of present study gave the following alpha helix predicted values 6.06%, 6.06%, 5.71% and 6.06% for CRP of patients with lung, breast cancer and CRP that retrieved from NCBI respectively. Secondary structure of present study gave the following β -turn predicted values 42.42%, 42.86% and 42.42% for CRP of patients with lung, breast cancer and CRP that retrieved from NCBI respectively. Secondary structure of present study gave the following coil predicted values 52.5%, 51.52% and 51.52% for CRP of patients with lung, breast cancer and CRP that retrieved from NCBI respectively figure(7).

For CRP of patient with lung cancer, out of all the templates given by PHYRE2 server, the

one with highest % i.d. (94 %) was used to predict the 3D structure. For CRP of patient with breast cancer, out of all the templates given by PHYRE2 server, the one with highest % i.d. (99 %) was used to predict the 3D structure. For CRP retrieved from NCBI, out of all the templates given by PHYRE2 server, the one with highest % i.d. (100 %) was used to predict the 3D structure figure(8).

For CRP of patient with lung cancer, out of all the templates given by swiss-model server, the one with highest % i.d. (94.53 %) was used to predict the quaternary structure. For CRP of patient with breast cancer, out of all the templates given by swiss-model server, the one with highest % i.d. (98.96 %) was used to predict the quaternary structure. For CRP retrieved from NCBI, out of all

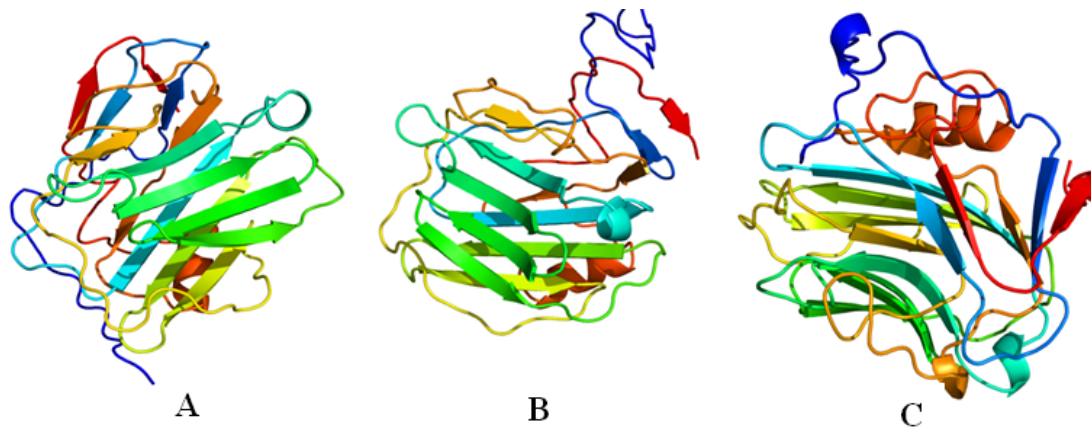


Fig. 8. 3D structure of CRP for patient with lung(A), breast cancer(B) and CRP retrieved from NCBI FASTA format-protein (C), by Phyre2 online software. The N-termini of proteins are colored blue and the C-termini red

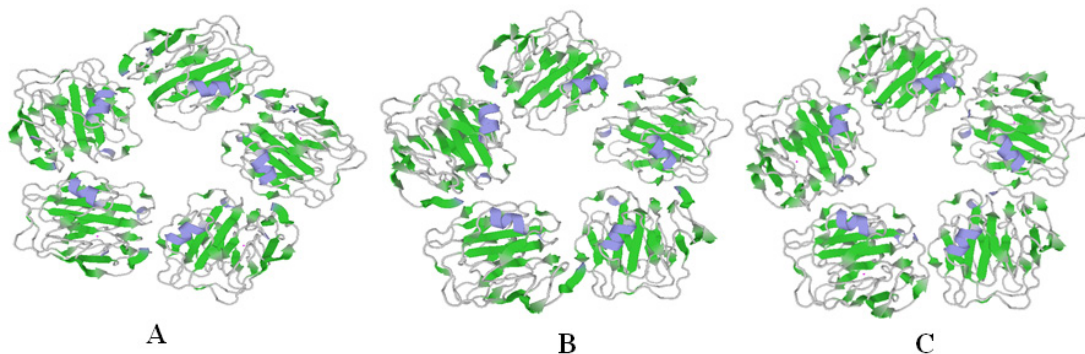


Fig. 9. Quaternary structure of CRP for patient with lung(A) and breast cancer(B) and CRP retrieved from NCBI FASTA format-protein (C), by swiss-model online software. The α -helix of protein is colored purple, β -sheets colored green and the coil light gray

the templates given by swiss-molde server, the one with highest % i.d. (100 %) was used to predict the quaternary structure figure(9).

The mutations on CRP gene for patients with lung and breast cancer were recorded by present study caused different effects on the structure of protein when it compared with CRP retrieved from NCBI as show in the result of structure analysis such as the numbers and positions of alpha helix, beta- turn and coil as shown in figures(7,8 and 4), this result agree with studies^{30,31} demonstrated that mutation cause large changes in the sequences this effected on the structurally of protein. Furthermore, this study has been shown that pathways of protein folding are largely unaffected by changes in the sequence.

CONCLUSIONS

In the present study, CRP is non-specific marker for patients with breast and breast cancer. The genotype at the site 159714441 was found to be associated with cancer disease. The mutations on CRP gene for patients with lung and breast cancer effect on the physicochemical properties of C-reactive protein such as molecular weight, PI, percentage of amino acid and also protein stability when compared with CRP retrieved from NCBI. The mutation effected on structures of CRP for the present study by changed the number and position of alpha helix, beta- turn and coil this effected on CRP by decrease $2Ca^{+2}$ ion for each subunit, this lead to loss the function of host defense of CRP for patients with lung and breast cancer.

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