

Early Diagnosis of Breast Cancer by Detection of Genetic Variation for BRCA1 and BRCA2 Genes in the Women's of Nineveh Province

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Abstract :

Women's Breast Cancer ranks first among other type in Iraq and the world. It often detects because of examinations performed in patients on familial cancer history. Approximately, 10% of hereditary breast cancer represents of all breast cancer cases and BRCA1/2 represented the responsible genes. Mutations in the BRCA1/2 germ increases the risk of hereditary breast cancer twenty times more. Therefore, the detection of BRCA1/2 genes mutations is important for the diagnosis, treatment and application of clinical methods of patients. New generation sequencing is more useful than classical methods. With this method, a patient-specific clinical treatment can be customized, treatment, and early diagnosis, facilitates new discoveries, identification of existing mutations, primarily breast and ovarian cancer.

In this study, the female patients had selected between the ages of 18-49 living in Nineveh-Iraq and having demographic identical qualities, the samples taken within the consent of the participants were examined and some significant mutations had been detected. Bioinformatics studies of variants evaluated as pathogenic were performed. In addition, variants of unknown clinical significance were identified and listed. Based on the results obtained in the study, it is concluded that tests should be applied directly in the laboratory environment to develop an understanding based on the Next Generation Sequencing approach for the detection of BRCA1 and BRCA2 mutations and to evaluate the functional role of the mutations. In addition, our study draws a conclusion for the early diagnosis of the New Generation Sequencing technique and the development of a personalized, patient-specific treatment approach.

Keywords: BRCA1, BRCA2, Next Generation Sequencing, Breast Cancer, Hereditary Breast Cancer.

1. Introduction

Woman's breast cancer can be considered the common type of cancer that Ninova sector suffered from, it is most probably comes from different risks factors such; as genetic characteristics, obesity, menopause, alcohol. Considering the time period from 1980 to 2010, Breast cancer ranks first among the cancer types diagnosed in Nineveh with 3563 cases and a rate of 15.3%. This study focuses on genetic factors that rank first among factors affecting breast cancer. So much so that, with an error in DNA repair, polymorphism and differentiation in gene function can have an initiating effect on cancer formation (Maluf, 2018).

Any disruption in the DNA structure will cause the DNA strands to separate. The resulting breakage is repaired by the BRCA1 and BRCA2 genes, known as breast cancer susceptibility genes. However, these two genes carry out the transcriptional regulation function together. BRCA 1 is located on the 17q chromosome and the protein of this gene consists of 1863 amino acids and 24 exons. The BRCA 2 gene is located on the 13q chromosome, but the protein of this gene contains 3418 amino acids and 27 exons. (Miki, 1994). BRCA 1, which is one of these genes that are inherited in families or by mutations occurring in the germ line, reveals a risk of breast cancer at a rate of 5-10%, while BRCA 2 constitutes a risk of breast cancer at a rate of 20-40% (Kuchenbaecker, 2017).

Screening for the detection of mutations or variations in these genes that repair breaks in the DNA helix through homologous recombination mechanism are important for early diagnosis of breast cancer. However, the presence of a similar case in the medical history of the patient's family increases the need for evaluation. Therefore, this study aims to evaluate the early diagnosis of breast cancer with the analysis and analysis of BRCA 1 and BRCA 2 genes in patients living in Nineveh, Iraq, who are at risk of breast cancer and who are admitted to the hospital with a specific complaint or for screening.

The world breast cancer cases approximately about 8-10%, this rate exceeds 15% in Nineveh, Iraq. It has not yet become widespread to address two gene structures, such as BRCA 1 and BRCA 2, among the methods preferred in breast cancer screenings performed today. However, when the literature is examined, it is seen that the

importance of BRCA 1 and BRCA 2 in this context has started to be understood very recently. BRCA 1 and BRCA 2 genes are not frequently examined in examinations for patients diagnosed with or suspected of having breast cancer in Iraq, and adequate studies are not conducted in this context. In this study, as a result of careful consideration of the genetic variations on BRCA 1 and BRCA 2 genes, clinical samples to be taken from women in Nineveh, Iraq will be examined and the result obtained will make a scientific contribution to the literature. In this context, the academic study we will carry out is aimed to contribute to the literature and researchers (Foulkes, 2016).

1.1. BRCA1 and BRCA2 Genes

Many previous investigations proved that the gene factor caused of this type of neoplasm such as BRCA1 and BRCA2 genes, these genes presenting a great function of tumor suppressor genes in the most cell and its sequence is responsible of happen of hereditary syndromes called HBC-SS (Hereditary Breast Cancer Site Specific) or HBOC (Hereditary Breast Ovarian Cancer) syndrome, which manifests themselves as breast and / or ovarian cancer. The probability of BRCA1 or BRCA2 genes mutations increases with increasing for both breast and / or ovarian cancer occurring of 45 - 65% of mutation type (Antoniou et al., 2003).

Basically, Inherited syndromes caused by BRCA1 or BRCA2 gene mutations are associated with a variety of clinical symptoms in the patient that may require a complex molecular analysis and considered the main diagnosis for both of breast and / or ovarian cancer in adults under the age of 45 in addition to the familial identification of first and second-degree relatives on a pedigree (Mohamad et al., 2008). However, it should be noted that molecular scans performed on the majority of breast cancer patients show that approximately 50% of patients with BRCA mutation do not have a family history (Ban & Godellas, 2014).

Genetic testing is a necessary condition for evaluating the presence of mutations in the BRCA1 and BRCA2 genes in female patients diagnosed with breast cancer under 35 years of age. However, the examination should also be performed on all women under 40, called triple negative patients, which are characterized by tumor overexpression of estrogen, progesterone, and HER2 receptors. Genetic consultation is strongly recommended for patients with bilateral breast cancer and a family history of breast and ovarian cancer, and especially for male patients diagnosed with breast cancer and also breast cancer, especially in cases of tumor formation under the age of 50 (Young et al., 2009).

1.2. Molecular Mechanisms of BRCA 1 and BRCA 2 Genes

Although germline mutations on the tumor suppressor genes BRCA1 and BRCA2 are known to cause breast and ovarian cancer, it has been demonstrated that these types of cancer are autosomal dominantly inherited (Hu et al, 2018).

Due to genotoxic stress during cell division, rupture of DNA double strands can occur and reduce the risk of insufficient genetic information through the cell cycle checkpoints S and G₂, BRCA1 and BRCA2 genes. The cell cycle is controlled by the BRCA1-RBBP8 complex through the activation of CHEK1 in the G₂ / M phase (Mussil et al., 2019, Simhadri et al., 2019, Takaoka et al, 2018).

Protein transcription from the BRCA1 and BRCA2 genes is responsible for repairing these breaks by homologous recombination. Homologous recombination is a type in which the nucleotide sequence changes between sister chromatids. In response to DNA damage, BRCA1 regulates RAD51, whose localization and function are controlled by BRCA2 during homologous recombination (Zhao et al., 2019, Petermann, 2018).

The protein transcript of this gene generates BASC through sensors for damaged DNA, other tumor suppressors, and signal transducers. Complexes of RNA polymerase II and HDAC combine with BASC. DNA repair is activated by activating the cell's response through polyubiquitin chains affected by the E3 ubiquitin-protein ligase. This ligase is also responsible for the tumor suppression of BRCA1. Genomic stability is provided by the BRCA1-BARD1 complex by various means such as transcriptional regulation, DNA repair and ubiquitination (Magdefrau et al., 2019, Stewart et al., 2017, Shimada 2018).

2. Materials and Methods

The clinical samples analyzed from female patients diagnosed with risk of breast cancer using a new generation sequencing technology, a multi-gene panel has been created for the diagnosis and detection of genes that cause breast cancer based on familial factors, such as BRCA 1 and BRCA 2, which are more effective in DNA repairs.

2.1. Choice of Patients

Experimental studies were carried out in the laboratories of the gynecological oncology unit of the research and practice hospital in Nineveh, Iraq. The patients were informed by obtaining ethics committee approval and consent voluntary statements. A criterion was considered in the selection, and the participants meet at least two of below:-

- 1- The diagnosis of Breast Cancer almost at 40 years old.
- 2-The presence of invasive and / or bilateral breast cancer and / or multiple organ cancers at any age.
- 3- Having a family history of breast cancer.

- 4-A first-degree female relative who is 40 years old or younger diagnosed with breast cancer.
 - 5-First-degree female relative diagnosed with primary breast cancer before age 50.
 - 6-Two first-degree relatives or one first-degree and one second-degree relative diagnosed with breast cancer at any age.
 - 7-Three first-degree or second-degree relatives (from the same side of the family) diagnosed with breast cancer at any age.
 - 8-One or more relatives diagnosed with cancer on the same side of the family (mother's or father's side).
 - 9-The fact that the patients are settled in the province of Nineveh, Iraq, and spent all or most of their life (more than half of their life) in this city.
- After the patients read, understand and approve the consent statement, blood plasma samples were taken at a maximum of 5 mL and EDTA tubes were kept at + 4oC.

2.2. Genomic DNA Isolation

Isolation of genomic DNA was carried out using Nucleon BACC3 Genomic DNA Extraction Kit (GE Healthcare, Life Sciences) from 5 - 10 mL peripheral blood sample. Nano Drop 2000 Spectrophotometer (Thermo Scientific) was used for quantitative examination of samples. In the DNA Isolation process, the instructions of the kit manufacturer were applied.

2.3. Qualitative and Quantitative Determination of the isolated gDNA

The measurement was made using the spectrophotometry device, 1µl In order to determine the concentration and purity of the samples after calibrated. Optical density value in the range of 260 nm - 280 nm was taken as the optimal value to determine the purity level of the sample. Results obtained in the range of 1.7 - 1.9 in the measurement of optical densities of 260 nm - 280 nm showed that the clinical samples obtained from the patients were at the desired purity.

2.4. BRCA2 N273H Single Nucleotide Polymorphism

Before performing the next generation sequencing process, the BRCA2 N273H single nucleotide polymorphism was studied and the result was noted in order to understand the quality of the BRCA2 gene and to provide preparation and sample source for the next generation sequencing stage.

Genotyping was performed using real-time PCR (polymerase chain reaction) in order to detect single nucleotide variation. In PCR, a DNA polymerase enzyme resistant to high temperatures increased the amount of DNA in vitro. Quantitative PCR was preferred for the simultaneous quantification of amplification reactions. At position c.1114 in exon 10 of the BRCA2 gene, the nucleotide of adenine is replaced by the cytosine nucleotide, resulting in an amino acid change from asparagine to histidine.

A TaqMan test (C_____807154_20) was used to detect BRCA2 transversion. It is noted that in the sequence VIC indicates nucleotide A while FAM indicates nucleotide C in the inversion N [AAT]> H [CAT].

Under the condition of real-time PCR reaction, this process was carried out with a 7500 cycle Applied Biosystem PCR device. Its plates are filled with the mixture containing the said ingredients. Plates were put into the device protected from light. Necessary thermal conditions have been observed.

2.5. Western Blot (WB)

The acronym (WB) refers to the Western Blot technique, it is considered a powerful biological experimental tool to explain the complex signaling events used to understand many biological processes and disease-related phenomena. There are many types of WB related to reagents on the market.

The experimental technique commonly used to detect expression of the target protein is shown as follows; The primary function of the known gel (SDS-PAGE) is to transfer the separated proteins to the membrane (eg PVDF membrane). The target protein is then discovered by the specific antibody that detects the target protein, and the specific antibody is recognized by the labeled second antibody. The WB technique is an experimental procedure. It has been used to detect protein expression in biology.

However, because of the wide variety of reagent sources, operating procedures, laboratory analysis experience, many WB experimental results are not compatible with the published article. For many years, it has been observed that Elab science has focused on immunology and accumulated a lot of experience in WB operation. it is the reason to make Western Blot kit is available to help researchers to find solutions for each case. Reagent kit provides the conventional WB experiments for all protein extraction, which offers simple operation, high detection sensitivity, low background, and strong stability. And Elab provides description, technical support, In addition to convenience and guarantee for obtaining the best Western Blot results ((Dennis-Sykes, C. A., et al., 1985, Heidebrecht, F., et al., 2009).

2.6. Next Generation Sequencing

The following steps were applied in the study conducted on 50 female patients living in Nineveh, Iraq in order to determine the genetic variation in BRCA1 and BRCA2 genes using the new generation sequencing method.

2.7. gDNA Measurement

In order to use the gDNAs isolated from the clinical samples of the patients in the next generation sequencing panel, a suitability check was carried out before measurement. Conformity control was carried out with the

Qubit's DNA HS brand and model experimental study kit manufactured by Invitrogen company of USA origin. Vortexing was applied before the samples were measured with the Qubit. For each sample to be used in the experiment, the working solution was formed by mixing 1 μ l of Qubit reagent and 199 μ l of Qubit Buffer solution. Vortexing was carried out by adding 2 μ l of isolated DNA to 198 μ l volume of the obtained solution. As a result of the measurement, each DNA sample was diluted and the density of 10 ng / μ l was adjusted.

2.8. Library Preparation for gDNA

The ION Ampliseq Library Kit 2.0 library kit produced by Thermo Fisher Scientific, USA, was used in the library preparation for gDNA. Samples were processed separately by separating the two pools in the panel containing 386 amplicons and 2 wells.

BRCA MASTR v.2.1 test kit (Multiplicom) was used to create the DNA library of amplicons and the manufacturer's instructions for use were followed step by step and applied.

At this stage, a multiplex PCR with five reactions including exons for each patient and a 50 ng gDNA template were used to process about 50 base pairs of neighboring regions of intronic genetic regions. After amplification, a 1: 1000 dilution of the purified multiplex PCR products was performed and re-amplified using specific molecular identification adapters (Multiplicom MID) for each patient. Amplicons in the second PCR were purified and purified using Agencourt AMPure XP (Beckman Coulter). Following this step, amplicons ranging in length from 350 to 500 base pairs were subjected to quality and quantity controls using the Bio-Rad Analysis kit. Equimolar concentration solutions of the five PCR outputs were pooled to create a BRCA amplicon library specific to each sample to create the sequencing master library. Subsequent clonal amplification of the resulting libraries was performed by emulsion PCR (emPCR) using the GS FLX Titanium emPCR kit LibA MV (Roche) according to the manufacturer's instructions. Subsequently, samples from a total of 60 patients were collected in 2 regions of a PTP well and sequencing was performed by performing sequencing. 454 GS FLX (Roche) was used in sequencing of the library.

2.9. Fragmentation

To digest the samples combined on an Eppendorf 96-well plate, the plate was taken on ice and run on ice throughout the procedure. After adding 2 μ l - 2.5 μ l of FuPa Reagent on the PCR end products on the Eppendorf 96-well plate, vortexing was performed and the precipitate was obtained.

2.10. Barcoding

IonXpress kit produced by Thermo Fisher Scientific, USA, was used to create barcodes for binding. Barcode content is as follows: Ion P1 - 2 μ l, IonXpress Barcode variable, 4 μ l distilled water.

2.11. Binding (Ligation)

The binding of the barcodes was carried out on ice, and the following reagents were added to the last sample separately for each patient, and binding was performed in the presence of ligase enzyme: Switch Solution - 2 μ l, Barcode Mix - 2 μ l. The enzyme was added to the isolated DNA sample on the Eppendorf 96-well plate and precipitated by vortexing.

2.12. Purification (Purification)

Samples were purified using Agencourt AMPure XP reagent and Eppendorf 96-well plate manufactured by Beckman Coulter, a US company. After the reagent was vortexed at 25 oC, the PCR mixture and Amplification Primer Mix in the ION Ampliseq Library Kit 2.0, produced by Thermo Fisher Scientific, USA, was placed on ice. After this step, a 70% ethyl alcohol solution was prepared and an equal volume of AMPure XP reagent was slowly added to each well for the samples on the Eppendorf 96-well plate and the wells were protected from light and air. After this step, it was kept at room temperature by vortexing for 15 seconds at 1500 rotations per minute. The Eppendorf 96-well plate was left on the magnetic stand to remove the supernatant, and the previously prepared ethyl alcohol solution was added to each well. Following the washing step, the supernatant portion was separated. After the application of ethyl alcohol was repeated, washing was carried out once more and the Eppendorf 96-well plate was again held at room temperature and on the magnetic stand. NcoI enzyme was used, which performs enzymatic digestion of gDNA used for the control of the patients' amplified cDNAs and enzyme functionality.

In the reaction, 0.5 μ l NcoI enzyme was used for 1 μ l of DNA and added to 5 μ l enzyme buffer without BSA. The DNA fragments were then analyzed with DNA Chip 1 K (BioRad).

Table 1 Primer list to verify the splice variant in the BRCA1 cDNA

	Primer Dizlemesi	Uzunluk (BazÇifti)
BRCA1_cDNA_fw	CAACATGCCACAGATCAAC	885
BRCA1_cDNA_rw	AATTCCTCCCAATGTTCC	885
BRCA1_intr21_fw	CCCACCCCTGTAATCACAAC	472
BRCA1_intr21_rw	GATCCCCAGGAAGGAAAGAG	472
BRCA1_intr21_ctr_rw	TCCCTCCCCCTCCTCTCTGT	1417

Table 2 PfUltra amplification protocol

Sıcaklık	Döngü
95° x 5'	1
95° x 30'' 55° x 60'' – 0,5°/döngü 72° x 65''	14
95° x 5'	1
95° x 30'' 55° x 60'' 72° x 65''	25
72° x 5'	1
4°	∞

3. CONCLUSION AND DISCUSSION

More than two thousand mutations have been identified in the BRCA 1 and BRCA2 genes, including deletion, insertion, and single nucleotide changes in coding sequences. It is known that the most common mutations are small insertions and deletions and the formation of dysfunctional BRCA proteins. The causes of mutations differ, and it has been reported that different mutations are observed even in a closed environment (Karami et al., 2013).

BRCA1 and BRCA2 mutations, known to be causal, were observed in 1/8 of the Nineveh female patients whose samples were studied within the scope of the study (Table 4.1) It is known that the participant patients with mutations in the BRCA1 and BRCA2 genes have at least one cancer history in their family.

However, the detected mutations; There are also cases of elaboration such as silent (synonymous) mutations, missense, double and triple mutation. These variants were found to be benign and of no clinical significance by the SIFT Sequencing tool.

A non-harmful variant was found in only one of the participant patients in whom breast cancer was evaluated as an early diagnosis. However, although these variants are thought to be harmless in the first estimates, considering that these variants are not found in healthy alleles, more comprehensive and different studies and approaches are needed to understand the roles and effects of these variants in the disease.

In addition, a possible mutation at the c9976A> T position on the BRCA2 gene is predicted to affect protein function. The result obtained by studying samples of another patient is that the mutation at the c.536A> G position is effective. In the same error, considering the mutation in the c.1075G> A position on BRCA2, it was seen that the mutations in question were previously registered in the database and this was thought to be effective in the development of the disease.

Multiple mutations were detected in two participants who had more family history of cancer in first, second, and third degree relatives than other participants. In order to ensure this situation, family members should also be tested with Sanger sequencing. C.5237A was identified in a patient with multiple cancer cases in the missense variant family at position > C and under surveillance with a diagnosis of early diagnosis / suspected cancer. It is evaluated that the variant is located on BRCA1, but will be effective in binding BRCA2 and BRIP1 proteins. Therefore, it is thought that the protein changes caused by the variant affect the function of both BRCA genes. At this point, the genetically unstable structure that occurs due to mutated proteins on the BRCA2 gene constitutes the basis of the instabilities and problems seen in the translation stage or in the post-translation stage. However, the problem is considered to lead to the formation of clones leading to dysfunctional reactions.

3.1 Polymorphisms with Undetermined Pathogenicity (VUS) Variants

Within the scope of our study, variants and polymorphisms with uncertain pathogenicity or of unknown clinical significance (VUS) were detected. Details of these polymorphisms are as follows.

Table 3 BRCA 1 Polymorphism

Gene	Exon	HGVS ¹ cDNA	BIC Designation	HGVS ¹ Protein	Mutation Type	Clinically important (BIC)	Reference number NCBI
BRCA1	11	c.2077G>A	D693N	p.Asp693Asn	Missense	No	rs4986850
	11	c.3113A>G	E1038G	p.Glu1038Gly	Missense	No	rs16941
	11	c.3548A>G	K1183R	p.Lys1183Arg	Missense	No	rs16942
	16	c.4837A>G	S1613G	p.Ser1613Gly	Missense	No	rs1799966

Table 4 BRCA1 VUS

Gene	Exon	HGVS ¹ cDNA	BIC Designation	HGVS ¹ Protein	Mutation Type	Clinically important (BIC)	Reference number NCBI
BRCA1	8	c.442-3_442-3delT	IVS7-3delT	-	Intervening Sequence	Unknown	n.r. ¹
	9	c.591C>T	C197C	p.Cys197Cys	Synonymous	Unknown	rs1799965
	10	c.671-12_671+12delT	IVS10+12delT	-	Intervening Sequence	Unknown	n.r. ¹
	11	c.1067A>G	Q356R	p.Gln356Arg	Missense	Unknown	rs1799950
	11	c.2082C>T	S694S	p.Ser694Ser	Synonymous	Unknown	rs1799949
	11	c.2311T>C	L771L	p.Leu771Leu	Synonymous	Unknown	rs1694011
	11	c.2612C>A	P871L	p.Pro871Gln	Missense		rs799917
	11	c.3119G>A	S1040N	p.Ser1040Asn	Missense	Unknown	rs4986852
	11	c.1911T>C	T637T	p.Thr637Thr	Synonymous	Unknown	n.r. ¹
	11	c.3418A>G	S1140G	p.Ser1140Gly	Missense	Unknown	rs2227945
	11	c.3711A>G	I1237M	p.Ile1237Met	Missense	Unknown	rs80357388
	13	c.4308T>C	S1436S	p.Ser1436Ser	Synonymous	Unknown	rs1060915
	16	c.4837A>T	S1613C	p.Ser1613Cys	Missense	Unknown	rs1799966

Table 5 BRCA 2 Polymorphism

Gene	Exon	HGVS ¹ cDNA	BIC Designation	HGVS ¹ Protein	Mutation Type	Clinically important (BIC)	Reference number NCBI
BRCA2	10	c.1114C>A	H372N	p.Asn372Asn	Synonymous	No	n.r. ¹
	10	c.1365A>G	S455S	p.Ser455Ser	Synonymous	No	rs1801439
	10	c.1151C>T	S384F	p.Ser384Phe	Missense	No	rs41293475
	10	c.865A>C	N289H	p.Asn289His	Missense	No	rs766173
	11	c.3396A>G	K1132K	p.Lys1132Lys	Synonymous	No	rs1801406
	11	c.4563G>A	L1521L	p.Lys1521Lys	Synonymous	No	n.r. ¹
	11	c.3807T>C	V1269V	p.Val1269Val	Synonymous	No	rs543304
	11	c.5199C>T	S1733S	p.Ser1733Ser	Synonymous	No	rs28897734
	11	c.4585G>A	G1529R	p.Gly1529Arg	Missense	No	rs28897728
	11	c.5312G>A	G1771D	p.Gly1771Asp	Missense	No	rs80358755
	11	c.6513C>G	V2171V	p.Val2171Val	Synonymous	No	n.r. ¹
	14	c.7242A>G	S2414S	p.Ser2414Ser	Synonymous	No	rs1799955
	27	c.9976A>T	K3326X	p.Lys3326Ter	Nonsense	No	rs11571833

Table 6 BRCA2 VUS

Gene	Exon	HGVS ¹ cDNA	BIC Designation	HGVS ¹ Protein	Mutation Type	Clinically important (BIC)	Reference number NCBI
BRCA2	10	c.865A>G	N289D	p.Asn289Asp	Missense	Unknown	rs766173
	10	c.1124C>T	P375L	p.Pro375Leu	Missense	Unknown	rs80358409
	10	c.1909+12_1909+12delT	IVS10+12delT	-	Intervening Sequence	Unknown	n.r. ¹
	11	c.2229T>C	H743H	p.His743His	Synonymous	Unknown	rs1801499
	11	c.2971A>G	N991D	p.Asn991Asp	Missense	Unknown	rs1799944
	11	c.3515C>G	S1172W	p.Ser1172Trp	Missense	Unknown	rs80358600
	11	c.3824T>C	I1275T	p.Ile1275Thr	Missense	Unknown	rs80358625
	11	c.5744C>T	T1915M	p.Thr1915Met	Missense	Unknown	n.r. ¹
	14	c.7008-20A>G	IVS13-20A>G		Intervening Sequence	Unknown	rs81002903
	16	c.7397C>T	A2466V	p.Ala2466Val	Missense	Unknown	rs169547
	16	c.7806-14T>C	IVS16-14 T>C	-	Intervening Sequence	Unknown	rs9534262

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