Molecular study of *CDKN2B* Gene in Iraqi KIDS suffered with Leukemia

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Abstract
The objectives of this study are to determine the genetic variation and detection new mutation of CDKN2B in child with leukemia in Mosul city. Cyclin-dependent kinase inhibitor 2B (CDKN2B) and Cyclin-dependent kinase inhibitor 2A (CDKN2A) are two genes located adjacent to each other at INK4 locus in a stretch of about 80 kb. They encode tumor suppressor proteins p16INK4A and p15INK4B, respectively that inhibit
cell cycle progression by forming complexes with cyclindependent kinase CDK4 or CDK6, This study included (91) patient of an age group ranging from (1-15) years of reviews to the private pathological analysis laboratories in the city of Mosul for a period ranging from June to September of 2022, According to the study's results, the proportion of Kids with leukemia, who observed the wild genotype AA was largest (33%) and the proportion who observed the mutant genotype TT was lowest (24%), and the proportion of observing for the heterogeneous AT was (43%) comparison to healthy group

Key words:*CDKN2B* gene ,T- ARMS-PCR, Polymorphism, mutation and leukemia

Introduction

Childhood leukemia is a class of illnesses marked by a variety of immune phenotypes and genetic alteration[1], Childhood leukemia is the most prevalent cancer in kids, accounting for 31% of all cancer cases[2], several discoveries have raised the disease's survival rate to more than 80%, although those who survive still deal with long-term morbidities[3]
Clearly a good objective, the identification of causes and prevention/early intervention. Ionizing radiation and congenital genetic abnormalities such Down's, neurofibromatosis, Fanconi's anemia, and Bloom's Syndrome are two known causes that account for fewer than 10% of instances, respectively. In the past two decades, the disease's incidence has risen by around 1% year, with comparable rates of increase decades ago[4], suggesting that the disease's causes are likely to have become more widespread in society over the previous several decade[5,6]. The development of "common acute lymphocytic leukemia" (or CD19+, CD10+, B-cell cALL) peak in 2–6-year-olds is the most remarkable aspect of this time period. This peak is missing in other populations, such as those in Africa and India, and is ecologically linked to nations with better socioeconomic level. [7]
There are several subtypes of the illness, each with a unique phenotype and age incidence trend. The lymphoid and myeloid divide, with roughly 80% of leukemia's being lymphocytic, is the most inclusive division for phenol typing leukemia. 11q23 MLL gene rearrangements predominately appear in infant leukemia's (1 year of age) and The child's existence appears to be punctuated by precise times when mutations occur, and the mechanistic formation is likely the result of separate causes [8].
Identification of some of these is essential to the present illness categorization technique because the genetic and epigenetic abnormalities frequently seen in juvenile leukemia's are significant prognostic markers [9]. Like other malignancies, childhood leukemia results

from two or more molecular alterations in stem-like cells, which can divide while still being immature. [10]

Most leukemia's have a pre-B cell characteristic, which means that they display cell surface markers seen on healthy pre-B cells and seem to be clonal outgrowths of healthy pre-B cells that have been "frozen" at a certain stage of development. Less frequently occurring leukemia s have myeloid or T-cell ancestry. Leukemia's naturally have the ability to mobilize and extravasation in the circulation since they are blood cells. In addition to their immense ability for "blast-like" proliferation, precursor blood cells normally create 1011 cells per organism every day. These characteristics are one of the six "hallmarks of cancer." [11]

Leukemia's appear to require significantly fewer genetic aberrations than solid tumors, which must gain these spreading capabilities through genetic mutations, possibly because hematopoietic progenitors already possess these "cancer-like" characteristics. Researchers have been able to pinpoint the timing of the production of genetic aberrations during the lifespan of the child due to the genetic simplicity of leukemia, the early age of juvenile leukemia's, and the availability of preserved biological resources**.** Utilizing cutting-edge genetic technologies and preserved patient samples, it has been determined if the majority of the frequent genetic alterations in leukemia formed during the embryonic period. [12]

The leukemia-related mutations are inadequate to produce illness on their own. The most frequent translocations for ALL and AML, respectively, are TEL-AML1 and AML1-ETO. Studies utilizing cord blood from healthy newborns show that chromosomal translocations may happen in the general population at a rate of 1% or higher**.** [13,14]

An essential part of maintaining cell cycle progression is maintained by the cyclin-dependent kinase inhibitor that the CDKN2B gene encodes. [15]

The human genome contains the CDKN2A and CDKN2B genes on the P arm of chromosome 9. In reality, these genes prevent the cell cycle, making them tumor suppressors. added that the CDKN2 gene has 3 coding exons (exons 1, 1, 2, and 3). The CDKN2 gene prevents cells from proliferating too fast, acting as tumor suppressors. [16]

In addition, it was noted that the cyclin-dependent kinase inhibitor 2B (CDKN2B) gene contains two coding exons (E1 and E2) and is located at the Homo sapiens locus in chromosomal band 9 p21.3. It is related to p14ARF and p16INK4A. [17]

Two distinct transcript variants, p15 and p10, are encoded by the CDKN2B gene. The primary function of p15INK4B is to specifically target (CDK4) or (CDK6) protein kinase inhibitors, which are also important for cell differentiation and cellular senescence [18]. Acute myeloid leukemia, a group of clonal stem cell disorders, and Myelodysplastic syndromes are among the many diseases in which CDKN2B is frequently mutated or deleted. [19]

The aims of this study are to determine the genetic variation and detection new mutation of CDKN2B in child with leukemia in Mosul city

Materials and method

Case Study:

This study included (91) patient of an age group ranging from (1-15) years of reviews to the private pathological analysis laboratories in the city of Mosul for a period ranging

from June to September of 2022, and it was relied on these clinical cases of the disease to choose Samples . The samples were divided into two parts based on the biochemical results: The first group: this group included 20 kid among kids who did not suffer from any health problems and were considered as a control group, The second group: This group included 71 kids with leukemia, based on biochemical results..

Collection of Blood sample

5 ml of venous blood was withdrawn from these patient and divided into groups, the first part was placed in tubes containing EDTA anticoagulant to extract DNA, and the second group was placed in tubes free of any anticoagulant. The tubes were left for one hour until the blood clotted, after which a centrifugation was carried out for a period of (10) ten minutes at a speed of (3000) cycles/minute to obtain the blood serum on which the biochemical tests were conducted.

DNA extraction:

DNA was extracted from the blood of (71) patient with control groups who were subjected to this study, using the modified method presented by (Iranpur and Esmailizadeh., 2010).

Genotyping:

Tetra–ARMS-PCR Reactions:

The DNA concentration in all study samples is adjusted after being measured by biodrop by diluting them with TE buffer solution to obtain the required concentration for performing PCR reactions and was (25) ng/microliter for each sample. Four primers are added for each primer reaction (F-outer and R-outer) for the whole gene, forward outer-reverse inner for the normal allele, forward outer-reverse inner) for the mutant allele.

The PCR reaction mixture is prepared by mixing the nucleic acid of each sample and the primer designated for the mutations under study with the components of the master-mix in a 0.2-ml PCR-tube produced by the English by Biolaps Company. Mix in the Microfuge for a period between (5-3) seconds to ensure that the reaction components are mixed, Then, the PCR tubes were inserted into the thermocycler within the special program for each mutation, then the reaction product is injected into the pits of the prepared agarose gel, at a concentration of 2%, with the addition the Ladder DNA prepared by Biolaps Company, in one of the first holes, after which the samples are migrated Running the electrophoresis device for a period of 45 minutes, after which the bands are imaged using a gel-documentation device.

Determination of the genetic variation of the CDKN2B gene byTetra–ARMS-PCR technology

The presence of the A $\longrightarrow$T mutation for CDKN2B gene was detected by adding 4 µl (100 nanogram) of template DNA and 1 µl (10 picomol) of each mutation specific primer for CDKN2B gene mutation supplied by the Korean company Macrogen to the contents of the master mix The presence of the TT mutation of for CDKN2B gene by adding 4 µl (100 nanogram) of template DNA and 1 µl (10 picompl) of each mutation specific primer , which was designed by the researcher using Pimer 3 software and used For the first time on this gene, it was prepared by the Korean macrogen company, and it was added to the contents of the master mix. The final reaction volume was 20 µl [20].

Table 1: Shows the primers used to determine genetic variation at the locus (CDKN2B) using PCR technology.

Primer	Sequence	Band size	Annealing
F-outer	5′ CCCGGATAATCCACCGTTGGCCGTA3′	460 bp	67
R-outer	5′ CCCTTGGCCCAGCTGAAAACGGAATT3′		
F-inner	5′ GGCGCGCCTGGATTGCTGCT3′	376 bp	
R-inner	GTCCGCGCTAGGCGCTTTTTCACG3′ 5′	248 bp	

Table 2: Shows the program adopted in the ARMS-PCR technique to identify the mutation of CDKN2B gene**.**

No.	Stage	Temperature	Time	Cycle number
1.	Initial denaturation.	95.0.	5.0. min.	1
2.	Denaturation.	95.0.	45.0. sec.	35
3.	Annealing.	67.0.	1.0. min.	
4.	Extension.	72.0.	1.0. min.	
5.	Final extension.	72.0.	7.0. min.	1
6.	Stop reaction.	4.0.	5.0. min	1

The optimal temperature for the primer bonding in this reaction was determined using the Gradient program on the thermo cycler device; the gradient was (5) and the mean temperature was (59) C. The temperature of 59°C was used since it gave the best results. Then the PCR reaction was separated by 2% agarose gel**.**

Determination of nucleotide sequencing of amplified pieces using DNA sequencing technology:

The sequence of the nitrogenous bases of the gene was determined for the CDKN2B gene samples that were included in the study for the purpose of verifying the validity of the designed primer for the purpose of detecting the presence of any additional discrepancies in the gene. These genes were obtained using a 3130 Genetic Analyzer device supplied by the Japanese company Hitachi. These gene sequences were matched with the gene sequences documented in the National Center for Biotechnology Information NCBI, and the results were analyzed using BLAST program. [21]

Table 5: shows the primers belonging to the for CDKN2B gene and on which the DNA sequencing test was conducted.

Primer	Sequence
CDKN2B-08-F	5′CTGTTACTGGCTGGGGAACT 3′
CDKN2B-08-R	5′ TGTCTGCACCACTGGAGGTA3′

Complete Blood Picture test:

This study also include some Blood components parameters used as a kidney frailer markers like (WBC -NEUT -LYMPH -MONO -EO -BASO - RBC-PLT)

Result and discussion.

The results showed, as in Figure (1), the existence of a relationship between Iraqi Kids with Leukemia and the mutation of CDKN2B Gene, As shown in Table (4).

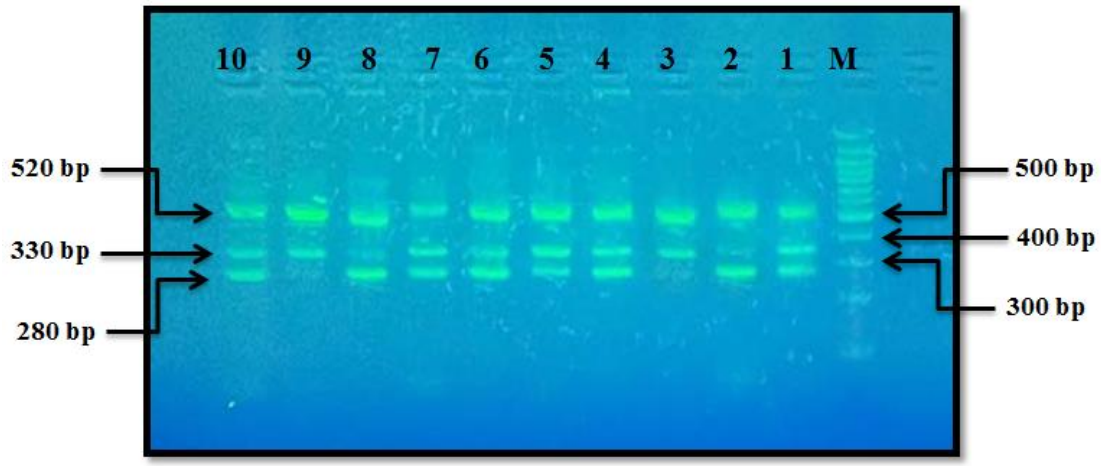


Figure (1): the relationship between Iraqi kids with leukemia and the mutation of CDKN2B Gene , with three band in PCR product 5200 bp fpr universal band, 330 bp for wild type allele and 280 for mutant type allele, samples (1,4,5,6,7,10) have hetero-genotype AT, samples (3,9) wild genotype AA and samples (2,8) have mutant genotype TT.

Table (4): Distribution of the percentage of allelic observations and genotypes of the CDKN2B gene mutation among a group of healthy groups and Iraqi kids with (leukemia), knowing that the A allele is the normal allele and the T allele is the mutant allele:

Genotypes	Patients		Control		P Value	OR	(95%Cl)
	NO.	%	NO.	%			
AA	24	33	16	80	P = 0.0331	5.6667	1.1488 to 27.9527
AT	30	43	2	10			
TT	17	24	2	10			
Alleles	NO.	%	NO.	%	P Value	OR	(95%Cl)
A	78	55	34	85	P = 0.0012	4.6496	1.8368 to 11.7694
T	64	45	6	15			

According to the study's results, the proportion of Kids with leukemia, who observed the wild genotype AA was largest (33%) and the proportion who observed the mutant genotype TT was lowest (24%), and the proportion of observing for the heterogeneous AT was (43%) comparison to healthy group, who observed the wild genotype AA was largest (42%) and the proportion who observed the mutant genotype TT was lowest (27%), and the proportion of observing for the heterogeneous AT was (31%), The allelic observation rate in Kids with leukemia was 45 percent for the mutant T allele and 55 percent for the normal A allele. The

percentage of the mutant allele T 45 seen relative to the control group was 15%, as opposed to 85% for the normal allele.

Determination of nucleotide sequencing of amplified pieces using DNA sequencing technology:

Figures (5) show the PCR reaction 270 bp, for CDKN2B-08 gene, on which the nucleotide sequence identification test was performed.

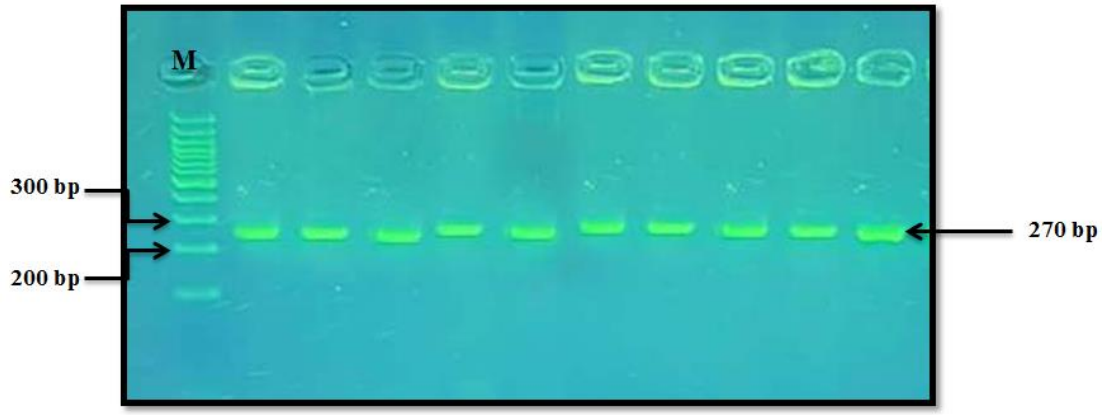
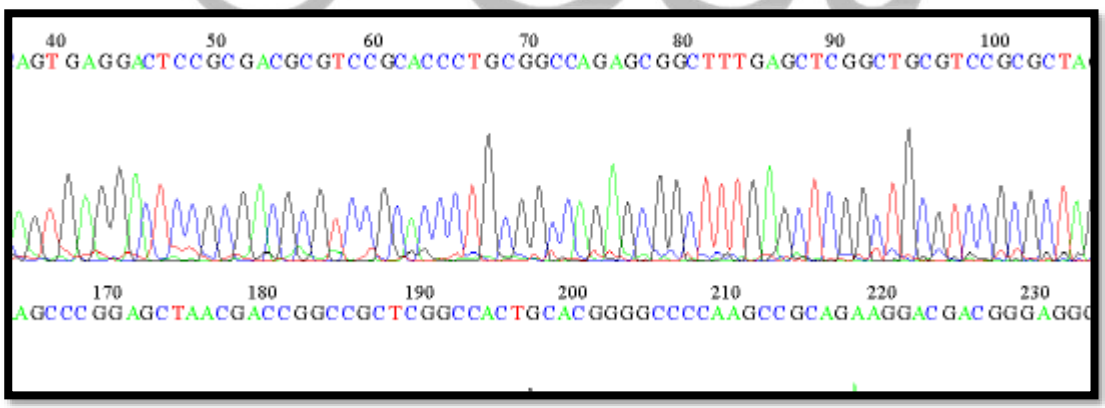


Figure 5: shows the PCR reaction product of CDKN2B gene in kids with leukemia and the reaction product 270 bp, M is the Ladder with a size of 100 bp, which was prepared by Biolabs and separated by 2% agarose gel.

The results of a sequencing test for the amplified of CDKN2B gene had differences in a number of nucleotides, however exon 5 had no differences. These findings are depicted in the following figures:



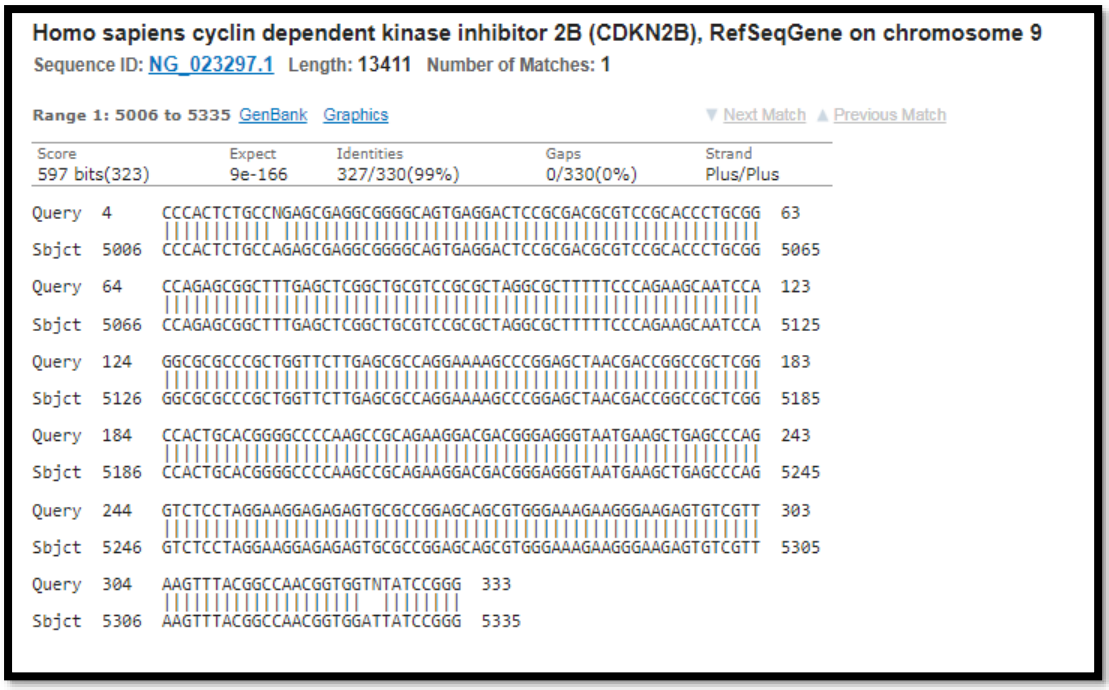


Figure 8: The result of matching with the nucleotide sequence of CDKN2B gene was compared with the sequences of the original gene at the NCBI site

Table 8: illustrates the locations and kinds of alterations in the *TP53* gene in breast cancer patients

ID sequince	Nucliotide	Location	Mutation type	Idendity	Gaps
(NG-023297.1)	A → T	(5326)	(Transversion)	(99%)	(0)
(AF513858.1)	A → T	(1642)	(Transversion)	(99%)	(0)
(NG-023297.1)	C → -	(5005)	(Deletion)	(99%)	(1)
(NG-023297.1)	A → C	(5017)	(Transversion)	(99%)	(1)
(NG-023297.1)	A → T	(5326)	(Transversion)	(99%)	(0)
(AF513858.1)	C → -	(1321)	(Deletion)	(99%)	(1)
(AF513858.1)	A → C	(1333)	(Transversion)	(99%)	(0)
(AF513858.1)	A → T	(1642)	(Transversion)	(99%)	(0)
(NM-004936.4)	A → T	(318)	(Transversion.)	(99%)	(0)
(NM-004936.4)	T → G	(319)	(Transversion)	(99%)	(0)
(NG-023297.1)	T → G	(5327)	(Transversion)	(99%)	(0)

(NM-004936.4)	A → C	(9)	(Transversion)	(99%)	(0)
(NM-004936.4)	T → G	(319)	(Transversion)	(99%)	(0)

When the table 8 is observed, it shows the different types of genetic variations and their locations on the CDKN2B gene after conducting a sequencing test and comparing them with the gene sequences at the NCBI site.

The results of this study shows an increase in the levels of Bloo parameters in kids with leukemia patients, WBC 2.54 (10^3\ul), NEUT 0.38 (10^3\ul), LYMPH 2.87 (10^3\ul), MONO 0.21 (10^3\ul), EO 0.2 (10^3\ul), BASO 0.02 (10^3\ul), RBC 4.5 (10^3\ul) and PLT 387 (10^3\ul) , compare with healthy group: WBC 7 (10^3\ul), NEUT 62 (10^3\ul), LYMPH 37 (10^3\ul), MONO 8 (10^3\ul), EO 2 (10^3\ul), BASO 0 (10^3\ul), RBC 3.2 (10^3\ul) and PLT 270 (10^3\ul).

Discussion

Leukemia is a group of diseases with varied immune phenotypes and genetic changes that progresses and is brought on by a variety of hereditary and environmental causes [20]. The purpose of this study was to look into any potential links between leukemia and the CDKN2B gene's rs3217992 polymorphism. We are unaware of the genetic contribution of CDKN2B locus variations in leukemia patients. Our findings showed a substantial link between the cdkn2b gene's T allele and leukemia, indicating that those who carry the T allele have a higher chance of acquiring the disease. Furthermore, we found that the CDKN2B rs3217992 T allele was more prevalent in leukemia patients compared to controls, proving that kids with the T allele of the rs3217992 polymorphism are more likely to acquire leukemia in our group., [21]A tumor suppressor gene called CDKN2B participates in cell signaling pathways and polymorphisms [22]

The INK4 locus on chromosome 9p21 has been linked to variations in children's vulnerability to leukemia. The current study is the first of its type in Iraq to examine the significance of genetic risk factors for leukemia in the CDKN2B (rs1063192, rs3217992) and CDKN2B-AS1 (rs2157719, rs4977756) genes situated at the INK4 locus. All cell types are affected by the cyclin dependent kinase inhibitor gene CDKN2B, which affects cell growth and senescence [23, 24]

Numerous studies show that CDKN2B single nucleotide polymorphisms can change how the protein functions. [25]

However, in our population, T allele confers protection as leukemia cases were found to have significantly lower frequencies of the minor allele (T) of rs3217992 variant (39.77%) as compared to controls (44.93%) (p=0.045). In preliminary analysis, our findings are consistent with the previous findings. The same relationship was shown in leukemia, where patients' T allele levels were considerably lower than controls' (p=0.024) in respect to controls. This was contrary to the earlier discovery. [26, 27]

The CDKN2B-AS1 gene, which codes for an antisense RNA that controls the production of CDKN2A and CDKN2B by building transcript complexes with polycomb proteins, is another gene of relevance found at the INK4 locus. Negatively regulating the production of inhibitor

genes is the antisense region. It has been demonstrated that ANRIL controls CDKN2B via binding to SUZ12, one of polycomb protein complexes 2, and that depletion of ANRIL results in enhanced expression of the CDKN2B gene. [28]

The accompanying shift in cyclin dependent kinase may have an impact on the apoptosis of stem cells. The gene has garnered particular interest in Iraq because of its possible involvement in the death of steam cells. [29–30, 31, 32].

In conclusion, our research showed that the CDKN2B gene variants are linked to the emergence of leukemia in Iraqi children. Particularly, in our community, bearers of the T allele of CDKN2B gene are more likely to develop leukemia. Future research of people from various ethnicities backgrounds are required to examine the relationship between these polymorphisms and leukemia risk, taking into account the complexity of the disease pathophysiology and the range of genetic and environmental variables involved.

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