

Cloning and sequencing of *Cystatin C* (CST3) from Human's Tissues

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Abstract

Background: Cystatins have regulative and preventive roles on Cysteine Proteases which existing in all biologic fluids of the human body. The protein-coding genes in humans and mice are located on chromosomes 20. The purpose of the present study is cloning and sequencing of hman Cystatin Gene of human normal tissues isolated from Iranian samples for future expression and production of recombinant protein to design of ELISA kit for cystatin detection in patients as a substitute for current creatinin. **Methods:** In this research, total RNA was extracted from human cord, thyroid, breast and blood tissues and then cDNA was synthesized by RT-PCR method. The resulted fragment was cloned in *pET28a* vector and transformed into the *E.coli* DE3-BL21 and finally DNA sequence of the gene was revealed by sequencing and compared with the reference sequence. **Result:** Amplification of cDNA from different tissue revealed that best result came from new born cord sample comparing to other human tissues like kidney. Cloned gene was verified by restriction digestion, PCR and sequencing. **Conclusion:** Analysis of sequence from cloned gene revealed that there is only single deletion before primer location with 99% alignment to the reference gene. Amino acid analysis shows that protein is correct and can be used for expression and design of Elisa kit.

Keywords: Anti-protease, CST3, Cysteine proteases, Human chromosome 20, cystatin c Gene cloning

INTRODUCTION

Cystatin C is a 13 KD protein composed of 120 amino acid residues which produce in all nucleate cells of the body (Abrahamson et al., 1987). Cystatin C is derived from a pro-protein containing cystatin C and a peptide signal, being this signal peptide is emphasis on its secretory action and also extracellular preventive operation of this protein (Barrett et al., 1986).

Cystatin production rate is partly constant from two months old to seventy years old and proportionate to amount of Glomerular Filtration Rate (GFR). Unlike serum creatinine, Rate of its production measure is not affected with muscular mass, sex or strain and also is not consist of other creatinine limitations in evaluation. It is filtered easily from the glomerulus due to the small size and pure positive net charge, moreover, its concentration is equivalent to the plasma. This substance generally absorbed by proximal tubule and its appearance in the urine indicate a deficiency in proximal tubule.

Serum concentration change of the cystatin C can be used as an indirect indicator of the GFR. Contrary to the most of European hospital that this test was replaced instead of the serum creatinine since 2008, this analysis normally does not perform in our country because of being expensive and unavailability of the required materials to calibrating the cystatin c. most of the Chronic Kidney Disease (CKD) that are easily treatable, could not be detectable with serum creatinine, while they are easily recognizable using cystatin C marker. Cystatin C background, as a biological marker, come back to the 1961 when a small protein in kidney, urine and cerebrospinal fluids observed (Grubb and Lofberg, 1982). In 1982 Grubb and Lofberg could Purified this marker from urine and kidney and succeeded to sequencing its amino

acid as well. Besides, this group has discovered a high amount of this marker in the urine of the kidney patient (Grubb and Lofberg, 1979). At 1985 for the first time, Grubb suggested to consider the amount of kidney filtration as a confirmatory test (Simonsen and Grubb, 1985). Abrahamson illustrated existing cystatin C in biologic liquids of the human body have a preventive effect upon cysteine protease in 1986 (Abrahamson). Structural studies and sequencing of this polypeptide pronounce of existing 3 forms of cystatins in their second family. These three (3) forms are nominated cystatin S, cystatin SN and cystatin SA (Bell et al., 1981).

Studies was distinguished that most of reaction carried out by form of the S. this kind of cystatins consisting of 120 amino acid is well-known as cystatin C (Berg, 1989). This kind of preventive protein secreted in extracellular and prohibit tissue damage caused by proteases, its coding gene located on the short arm of chromosome 20 next to the others type 2 coding genes of cystatin C. This non-glycosidic protein has two (2) disulfide bands and constantly produced by all type of nucleate cells and consider as a measure for diagnosis of cerebral hemorrhage. In this disease, cystatin C is insufficient in order to its change in genomic sequence of bases or mutation occurrence, this conditions resulted in to the dependent arteries amyloid of the cystatin C (Grubb et al., 1984).

In 1987 Abrahamson and his colleagues cloned this gene from cDNA with enzymatic cutting by *Alu* enzyme and Restriction Fragment Length Polymorphism (RFLP). By examining the DNA of patients with cerebral hemorrhage they were found that in the nucleotide sequence of the gene mutation has occurred.

Cystatin c is a member of family of proteins with several same sequences and different operations, some of these proteins have a preventive and regulative role on cysteine proteases.

Cystatin C family is divided into three (3) below family:

- **stefins**: This family has intercellular expression
- **cystatins**: This family has extracellular expression
- **kininegens**: This family has extracellular expression

According to the above categories, cystatin C belong to the second group that have a role to prevent the cysteine protease enzyme that observe in biologic liquids, tissues and different points of the human body (Barrett et al., 1986; Lindholt et al., 2001; Turk et al., 1986) it is important to notice that cystatin C play its preventing role on the molecular forms of *papaein* and *legumin* in protease cysteine (Grubb et al., 1984; Alvarez-Fernandez et al., 1999). For this reasons and benefit of Cystatin C detection in medical diagnostics lab as a new marker for kidney disease, in this project we started to clone and sequense part of systatyin C Gene that further this protein will be expressed in *E. Coli* to design and develop ELISA kit in IRAN.

MATERIALS AND METHODS

For the purpose of this study, sets of primers were designed using software such as *primer3* and *blast*. As a human tissue for RNA extraction we used thyroid, breast and cord tissues that newly biopsied obtained from surgery division of baqiyatallah hospital. Tissue sample was powdered by liquid nitrogen and the RNA was extracted according to the method in the kit. Total RNA was extracted by the *tripure roche* solution and cDNA synthesized by *transcriptor first strand cDNA synthesis* from *roche* company.

The impurities were removed by washing the sample with 75% ethanol and centrifugation at 12000 rpm in 5°C that lead to observing the 28S, 18S, 5.8S components in 1.2% agarose.

The cDNA was approved by using the *control primer mix* pBGD, production of 151 bp PCR fragment approved the correct synthesis of cDNA.

By PCR amplification and using two specific forward 5'-agg-gat-cca-tgg-ccg-ggc-ccc-tgc-gcg-ccc- cg<c>-3' and reverse primer 5'-aca-agc-ttc-tag-gcg-tcc-tga-cag-gtg-gat-tt<c>-3' fragment was synthesized. The PCR reaction mixture (25 µl) contained 0.5µl cDNA as a template, 0.7 µl of each oligonucleotide primer, 0.5 µl dNTP mix, 2.5 µl of 10x *pfu* buffer, 2.0 µl of Mgso4 ,0.25 µl *pfu DNA polymerase* enzyme and 17.7 µl water, after preparation of above mixture, amplifications were performed using automatic *Bio Rad* Thermo Cycler with the following thermal cycling profile: an initial denaturation at 95°C for 10 min followed by 30 cycles at 95°C for 1min, annealing at 58°C for 1 min, extension at 72°C for 1 min and final extension period of 10 min at 72°C. PCR product was visible by 1.2% agarose gel, stained by Ethidium Bromide and visualized in gel documation system.

For cloning, Plasmid *pET28a* was extracted and purified by using the overnight culture of bacteria and plasmid was extracted by mini prep alkaline method (Birnboim and Doly, 1979). In according to the enzymatic map, the plasmid was cut by *Rsa I* resulting fragments of 1270-3030-4261 bps. Then plasmid and DNA fragment simultaneously was lied at the vicinity of the restriction enzymes *Bam HI* and *Hind III*, then ligation was performed by T4 DNA Ligase in 30 µl final volume, 2.5 µl T4 DNA Ligase, 8.5 µl plasmid, 10 µl DNA fragment, 3 µl buffer, 7.5 µl water and ultimately mixture was

put into the refrigerator for 24h in 6°C.

Transformation of the cloned plasmid *pET28a* containing cystatin fragment in the host cell, *E.coli* DE3-BL21 was performed by thermal method. Then cultured in liquid LB medium in 37°C for 1 hour, followed by cultivating in LB agar containing 100 mg/ml concentration of kanamycin and incubated in 37°C for 12 hours.

After incubation period correct colonies were surveyed for plasmids containing desired gene by restriction enzyme, PCR and sequencing method.

RESULTS

In this study, The total RNA was extracted from human thyroid, white blood globules, breast and new born cord tissues newly biopsied from patients undergoing surgery. The tissues were not cancerous and healthy. Result showed that after several attempt to extract RNA from different samples best result for total RNA extraction was from newborn cord and thyroid but for 2 other tissues, a slight amount of RNA was extracted but not useful for cDNA synthesis.

cDNA was synthesized in and approved by using the *control primer mix* pBGD, (figure 1). PCR with two specific primer resulted in correct fragment ready for cloning, (figure 2).

Cloning of the Cystatin C gene fragment was approved by several restriction digestion of *Bam* *HI* and *Hind* *III* and specific PCR. Finally PCR fragment and cloned gene was sent to the BIONNER Company for sequencing.

Resulted sequences were analysed and compared with reference sequence and gene bank database by bioinformatics software. The results showed that there is a 99% similarities and only single base deletion was observed before the start coding of the systatin C DNA that has not effect on resulted protein sequence. (figures 3 and 4). Therefore this protein can be used as a marker in future research for designing ELSA kit for laboratory diagnosis of kidney diseases (result not published)

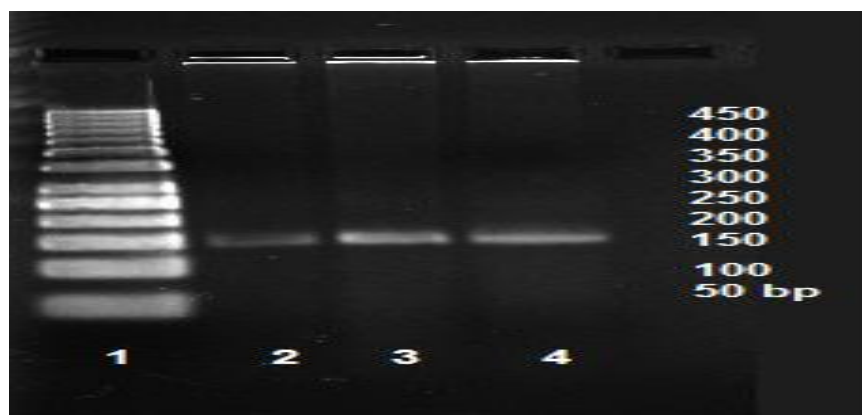


Figure1. PCR product produced using specific primer of PBGD: lane 1, 50 bp DNA ladder; lane 2 and 3, cord tissue; lane 4, thyroid tissue.

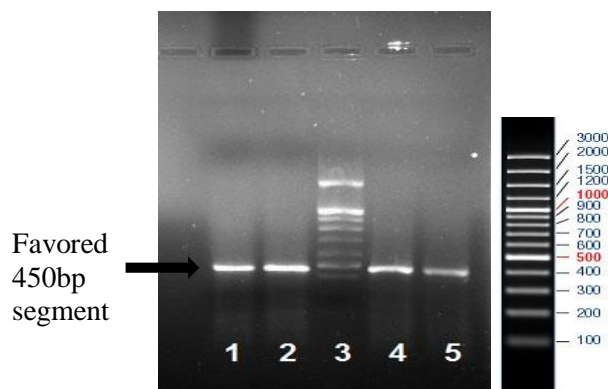


Figure2. PCR product (450 bp on 1.25% agarose gel: lane 1-2-4, cord tissues; lane 5, thyroid tissue; lane 3, 100bp DNA ladder

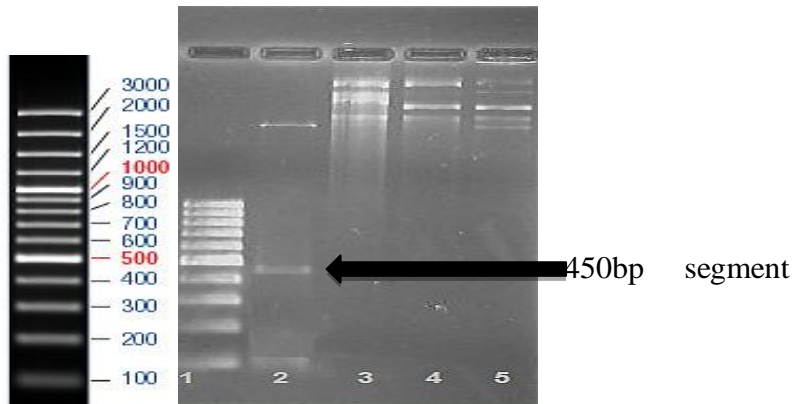


Figure3. Cloned Gene in the vector: lane 1, 100bp DNA ladder; lane 2, enzymatic cut off plasmid; lane 3, plasmid consisting genomic segment; lane 4 and 5, plasmid without segment

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Score = 750 bits (406), Expect = 0.0
Identities = 413/416 (99%), Gaps = 1/416 (0%)
Strand=Plus/Minus

Query 2 CAATGTAATTGTGCCCTGCC-AGGCACAGCGTAGATCTGGAAAGAGCAGAAATGCTTTCTCT
60
Sbjct 489 CAAGGTCATTGTGCCCTGCC-AGGCACAGCGTAGATCTGGAAAGAGCAGAAATGCTTTCTCT
430
Query 61 TTTCAGATGTGGCTGGTCATGGAAGGGGCGAGTTGTCCAAAGTTGGGCTGGGTCTTGGTACA
120
Sbjct 429 TTTCAGATGTGGCTGGTCATGGAAGGGGCGAGTTGTCCAAAGTTGGGCTGGGTCTTGGTACA
370
Query 121 CGTGGTTGCGCCACGCTCCACGTCCAAGAAGTAGTTACCCACGCTACGATCTGCTTGCG
180
Sbjct 369 CGTGGTTGCGCCACGCTCCACGTCCAAGAAGTAGTTACCCACGCTACGATCTGCTTGCG
310
Query 181 GGC CGCGCACCCACCTGCAGCGCGCGGCTGTGGTACATGTCGTTGCTGGCTTTGTTGTACTC
240
Sbjct 309 GGC CGCGCACCCACCTGCAGCGCGCGGCTGTGGTACATGTCGTTGCTGGCTTTGTTGTACTC
250
Query 241 GCGCACGGCAAAGTCCAGTGCACGCCGACACCCCTCCTCCTCCACGCTGGCGTCCATGGG
300
Sbjct 249 GCGCACGGCAAAGTCCAGTGCACGCCGACACCCCTCCTCCTCCACGCTGGCGTCCATGGG
190
Query 301 GCCTCCACACAGGCGCGCGGCTTGCCGGGACTGGAGCCGGCCGCGGGGCTCACGGCCAG
360
Sbjct 189 GCCTCCACACAGGCGCGCGGCTTGCCGGGACTGGAGCCGGCCGCGGGGCTCACGGCCAG
130
Query 361 GGCCACGGCCAGGATGGCCAGCAGGAGCAGCGGGGCGCGCAGGGGCCCGGCCATGG 416
Sbjct 129 GGCCACGGCCAGGATGGCCAGCAGGAGCAGCGGGGCGCGCAGGGGCCCGGCCATGG 74
    
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Figure 4. Comparison of DNA sequence from our results to the standard reference: sequence revealed 99% similarities

DISCUSSION

The best way to measure Glomerular Filtration Rate (GFR) is to surveying the kidney operation by injection of substances like Inulin and radioisotopes such as S1 chromium-EDTA, Icthalamate, 12SI, 99-MTC-DTPA or radioactive that create a contrast like Ichorel. These methods are complicated, expensive, time consuming and have a clinical side effects (Zahran et al., 2007; Roos et al., 2007).

Creatinine is a biomarker vastly used in diagnosis of kidney diseases, but when the kidney has a partial damage it is not an acceptable marker also its level changes with mass of muscle volume and others interfering factors. The formulas such as COCKROFT, GAVIT or MDRD tried to correct these interfering criterias (King and Levey, 1993). Kidney latent malady is a procedure that one kidney lose along with months to years. Kidney latent malady is consist 15% of healthy population in pandemic. Imposition expenses of this disease is about 1/3 of remedial expense in the USA and abundant articles was published of this case demonstrated a lot of disease that cannot be diagnosed by creatinine analysis (Premaratne et al., 2008).

The indeterminate range of creatinine is between 40-90 ml/min/ in 173m³, this range is where the GFR begin to

decrease and creatinine unable to detect it, while cystatin C analysis is more specific and is able to diagnose its decreasing. At this point creatinine has a False Negative result and cystatin C present to us an exact amount of GFR situation. In this study, due to the importance of this new marker, for kidney malfunctions we have tried to use several human tissues from Iranian patient to isolate total RNA, synthesize cDNA and to clone segment of cystatin C gene. We decided to clone this fragment with size of 450 bp in strong bacterial expression vector, plasmid *pET28a*. Resulted sequence was compared with the reference sequence of cystatin C by Abrahamson and his colleague. A 99% similarity exist to this gene, only one deletion observed before start codon with no effect on amino acid open reading frame. This work will followed with expression and purification of recombinant Cystatin C protein for design of ELISA kit for new diagnosis method in Iranian laboratories. Work is under way to accomplish this goal. (Result is not published)

This is for first time that human cystatin C gene is cloned and sequenced in Iranian.

CONCLUSION

For the first time in Iran we had studied molecular sequence of Cystatin C by isolating total RNA from several tissues biopsied from surgery patients. cDNA was synthesized and cloned in *pET28a* expression vector. Sequence resulted from cloned gene was analyzed and revealed its accuracy. This work is preliminary work that will be continued to express and purified desired recombinant Cystatin C protein to design and develop new diagnostic kit in Iran for kidney diseases.

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