Isolation And Cloning of Human NQO1 Promoter In Pgl3 Basic Vector

Asma Chinigarzadeh, Razauden Zulkifli, Iman Yaze and Reyhaneh rahnamai tajadod

Abstract— Malaria is a major public health problem caused by Plasmodium falciparum, a parasite that infects red blood cells. Recently, several polyphenolic compounds have been reported capable of preventing the progression of malaria parasite. This observation may be related to NAD (P) H: quinon oxidoreductase: a flavoprotein responsible for catalyzing two-electron reduction and detoxification of quinones and their derivatives. In this study the 2123 bp of the 5` upstream of the first transcription start site was successfully isolated. Based on the bioinformatic program, several regulatory regions such as Antioxidant Response Element (ARE) may be responsible for the direct regulation of polyphenols on this enzyme. It was predicted at -477 from the first transcription start site. Upon isolation, this fragment was used in a cloning process into the pGL3 Basic vector and transformed to E.coli DH5α competent cells.

Index Terms— NQO1, promoter, Transcription factor, Malaria, Polyphenols, pGL3 vector, E.coli DH5α, Cloning

1 INTRODUCTION

The focus of this study was the isolation of NQO1 gene promoter and its constructs. It was fulfilled in order to explore the effect of certain polyphenols in regulation of gene expression for future work. In this paper, four sections will be described. In the first section, malaria as an infectious disease has been introduced. In the second section, several kinds of polyphenolic derivatives and the effects of these compounds on this disease have been reviewed. In the third section, NQO1gene, promoter, and regulatory sequences are discussed. Finally, the effect of polyphenolic compounds in regulation of gene expression related to transcription factors will be discussed. Malaria is one of the most destructive and dangerous parasitic infections in many tropical and subtropical countries [1]. Almost half of the world's population lives under the risk of this disease. Malaria is caused by protozoan parasites of the genus Plasmodium. Four species of Plasmodium infect human and the most deadly is P. falciparum [9]. The infected Anopheles mosquito injects the sporozoites into human dermis. After inoculation, they migrate to liver cells and generate the merozoites. Then these cells invade erythrocytes (RBCs) and in this erythrocytic stage, a severe condition of malaria occurs. The life cycle is completed when a mosquito ingests gametocytes [5]. It has been proven that the malaria parasite generates a huge amount of reactive oxygen species (ROS) inside the infected erythrocytes. This is produced due to the oxidation and degradation of ingested hemoglobin in the acid environment of the parasite's food vacuole. ROS causes oxidative and electrophilic stress, also membrane and DNA damage. The most crucial function of defensive system in body is its ability to control oxidative stress [4]. Reactive oxygen species are detoxified by the antioxidant mechanisms [7], [8].

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Polyphenols are hydroxylated phenolic compounds extracted from plants. Some of them (e.g., flavonoids) are known for their antioxidant and antiparasitic activities [3]. The antioxidant actions of polyphenols are principally because of their redox properties, which play a critical function in neutralizing and adsorbing free radicals. Luteolin is one of the polyphenolic compounds in plants, which prevent the progression of parasite growth beyond the young trophozoite stage. Therefore, the parasites are incapable to complete a full intra erythrocytic cycle [3]. In addition, Elagic acid is found in various plant products with antioxidant activities. It has shown high activity in vitro against all Plasmodium falciparum strains and affects the parasite growth in the mature trophozoite and young schizont stages [6]. Some enzymes protect the cells against oxidative stress. One of these antioxidant enzymes is NAD (P) H: quinone oxidoreductase (NQO1). NQO1 is a flavoprotein that catalyzes detoxification and two-electron reduction of quinones as well as its by-products. It protects the cells against oxidative stress and redox cycling [2]. NQO1 gene expression is induced in response to some compounds such as oxidants, antioxidants, xenobiotics, UV light, heavy metals, and ionizing radiations [8]. Antioxidant response element (ARE) found in several gene promoter is activated by nuclear E2-related factors such as NF-E2-related factor2 (Nrf2). It is an essential transcription factor in the management of detoxification and antioxidant protection as well as overall regulation of the protective response. Nrf2 has the ability to manage many different aspects of cellular protection [4]. ARE-transcription factor complex is necessitated for both induction and expression of NQO1 and other detoxifying enzyme genes. In this process, Nrf2 with c-Jun bind to the ARE and activate the gene expression [2].

2 METHODOLOGY

2.1 Sequence Determination of NQO1 Promoter

For designing the primers, the sequence of human NQO1 gene and promoter were identified from National Center for Biotechnology Information (NCBI).

2.2 Designing the Primers for the First PCR and With Restriction Enzymes Cutting Sites For Second PCR

To isolate the promoter sequence, the primers were designed

using primer3 website (http://frodo.wi.mit.edu/primer3/) (Table1) . The primers were aligned with sequence of NQO1 promoter by using bioinformatic tools for assurance of primers correctness. The enzymes chosen in this project were *MluI* and *BglII* with cutting sites ACGCGT and AGATCT respectively in the 5` ends of forward and reverse primers. The poly A sites were introduced to protect the restriction sites from damage and help the enzyme to digest the ends. PCR was carried out

TABLE 1

Oligo name	Primer sequence	Length	Melting temperature	GC (%)
Primer-left	5`-TGTAACTACC GCCACCATCA-3`	20	57.80	50.00
Primer-right	5`-CCATCGTGAC ATTTTTGCAT -3`	20	53.70	40.00
Left primer with RE cutting site	5`-AAAAACGCG TTGTAACTACCG CCACCATCA-3`	30	64.77	46.67
Right primer With RE cutting site	5`-AAAAAGATC TCCATCGTGAC ATTTTTGCAT-3	30	59.30	33.33

Primer sequences for isolating human NQO1 promoter region and for introduction of restriction cutting sites

and the products extracted for digestion.

2.3 Isolation of Genomic DNA from Human Blood

Since mature red blood cells have no nucleus, DNA must be taken from either leukocytes, or immature and nucleated red blood cells. DNA extraction methods are universal, but some particular processes are necessary when working with blood, due to its delicate nature. Sample of human blood was taken and collected in EDTA tubes to prevent clotting and then the extraction process of DNA was done using Genomic DNA Purification Kit from Promega. The concentration and purification of DNA were determined by measuring the 260/280nm absorbance ratio. Then Agarose gel electrophoresis was carried out to identify DNA fragments.

2.4 PCR Amplification

First sets of primer were used to amplify the NQO1 gene promoter. The specific bands were excised and extracted from the gel using QIAgen QIAquick Gel Extraction Kit to use as DNA sample for second PCR process.

2.5 DNA Sequencing

PCR product was sealed and sent to Medigene laboratory for sequencing. The result was compared with the sequence obtained from NCBI by using BLAST tool.

2.6 Grow of *E.coli* on Plate and in Liquid Culture

Growing of *E.coli* on agar plates or in liquid medium was initiated by using a single *E.coli* colony derived from the glycerol stock after streaking on agar plates. In order to transform the plasmid into the host, cells first needed to be competent.

2.7 Preparation of PGL3-Basic Vector by QIAprep Spin Miniprep Kit

Single colony was picked from a freshly streaked agar plate and was immersed in 5 ml LB containing with 50 µg/ml ampicillin. The culture was incubated in shaking incubator at 37 °C/ 200 rpm overnight. After that, Plasmid was isolated using QIAprep Spin Miniprep Kit.

2.8 Double digestion of PGL3-Basic Vector and NQO1 promoter

The gel purified PCR product (NQO1 gene promoter) and plasmid were digested simultaneously using appropriate ingredients and conditions by Promega. Two enzymes with 100% compatibility in buffer “D”(Table2) were used.

TABLE 2

Enzyme	BufferSupplied	A	B	C	D	Multi-Core
Bgl II	D	25-50%	75-100%	75-100%	**100%**	<10%
Mlu I	D	10-25%	25-50%	50-75%	**100%**	10-25%

Percent Activity in 4-CORE® Buffer System of MluI and BglII restriction enzymes

2.9 Ligation with T4 DNA ligase

The digested insert (the NQO1 promoter) and PGL3 Basic vector were ligated with T4 DNA ligase using Promega kit. The solutions were mixed and incubated overnight at 4°C before being transformed into *E.coli*.

2.10 Transformation of ligation reaction into *Ecoli* DH5alpha competent

20 µl of the ligation reaction was transformed into 150 ml competent cells. Only transformed cells survived on the plates as they contained PGL3 with insert.

3 Result And Discussion

3.1 Sequence Identification of NQO1 Gene and Promoter

The sequence of NQO1 gene and promoter were determined using NCBI database. The sequence length of NQO1 gene (Accession number NG_011504) was 24230bp and promoter (AY426763) was 2123bp (Figure1). The sequence of NQO1gene was aligned with promoter sequence by ClustalW2 tool in European Bioinformatics Institute (EBI) websites. The specific forward and reverse primers were designed in primer3 Input (version 0.4.0) and aligned with NQO1 promoter using Pairwise Alignment website

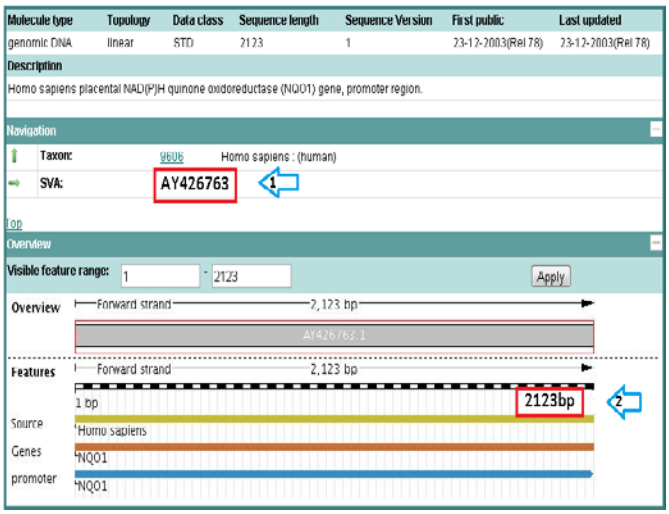

Figure1.Sequence of human NQO1 promoter. Number 1-2: Accession Number and length of NQO1 promoter

3.2 Characterization of Potential Transcription Factor Binding Sites within NQO1 Promoter

The regulatory regions related to transcription factors for NQO1 gene promoter was analyzed using Mat Inspector ® software (Genomatix) and Japanese bioinformatics (http://www.cbrc.jp/research/db/TFSEARCH.html)

3.3 Isolation of Genomic DNA from Human Blood Sample

Genomic DNA was extracted from fresh blood by Genomic DNA Purification Kit. 5 µl of DNA was transferred to agarose gel and the band observed above the 1kb DNA ladder (Shown Figure 2) as the whole of genomic DNA in human is approximately 3 billion base pairs.

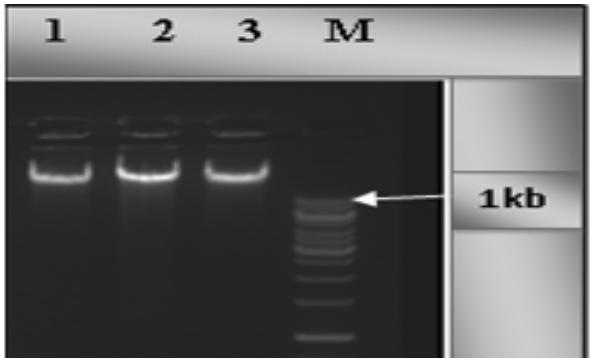

Figure2. DNA extracted from blood .Lane M: 1kb Marker, Lane 1-3: Genomic DNA

As mentioned above the measuring of DNA concentration and purification was carried out using Nanodrop Spectrophotometer. The OD260: 280 ratio of purified DNA is found in the expected range of 1.8-2 ng/µl with concentration 124 ng/ µl. An aliquot of 10 µl was transferred into the 10 sterile 1.5 ml microcentrifuge tubes and stored at -20°C for using PCR.

3.4 Polymerase Chain Reaction (PCR)

PCR was successfully done to access and isolate the exact region of NQO1 gene promoter (Figure3). The PCR product were sent for sequencing.

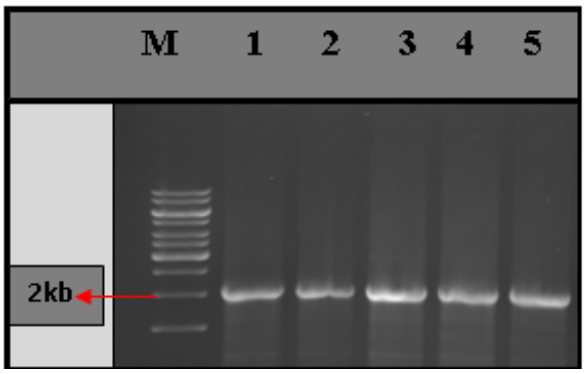

Figure3. PCR product of NQO1 promoter.M:1kb marker, Lane 1-5 promoter fragments

The result was analyzed with Sequence Scanner software V1.0. The nucleotide sequence obtained was submitted for similarity search in the GeneBank database using BLAST NCBI with high similarity with Homo Sapien NAD(P)H dehydrogenase, Quinone 1 (NQO1) (Figure 4).

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
NG_011504.1	Homo sapiens NAD(P)H dehydrogenase, quinone 1 (NQO1), RefSeqGene on chromosome 16	1690	2746	99%	0.0	93%
AC092115.2	Homo sapiens chromosome 16 clone CTD-2033A16, complete sequence	1690	6679	99%	0.0	93%
AY426763.1	Homo sapiens placental NAD(P)H quinone oxidoreductase (NQO1) gene, promoter region	1685	1685	99%	0.0	93%

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
NG_011504.1	Homo sapiens NAD(P)H dehydrogenase, quinone 1 (NQO1), RefSeqGene on chromosome 16	828	828	99%	0.0	92%
AC092115.2	Homo sapiens chromosome 16 clone CTD-2033A16, complete sequence	828	929	99%	0.0	92%
M81596.1	Human NAD(P)H:quinone oxireductase gene, exon 1	778	778	83%	0.0	95%

Figure4. Comparison between the isolated sequence and NCBI database for forward and reverse NQO1 promoter. The top and bottom panels show forward and reverse promoter sequence comparisons respectively.

3.5 Amplification of Promoter Using Primers with Restriction Cutting Sites

To insert DNA fragment into the vector, same primers with restriction enzyme binding sites were applied. Restriction sites for Mlu I and Bgl II were located in the 5` end of forward and reverse primer respectively. By using bioinformatic tools NEB

cutter (http://tools.neb.com/NEBcutter2/) it was confirmed that is no any restriction binding site inside the sequence for these enzymes; thus, they were good choices for cutting both ends of promoter from the gene. Figure 5 shows the amplified promoter with binding sites for Mlu I and Bgl II.

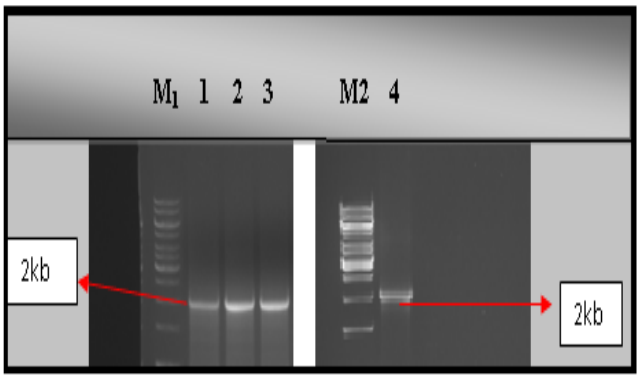

Figure5. Ampilfied promoter with RE sites, M1&M2: marker, lane1-3 before extraction, lane 4 after extraction.

PCR product with restriction enzyme binding sites was extracted from agarose gel to be digested for ligation into the PGL3 vector by T4 DNA ligase (Figure6).

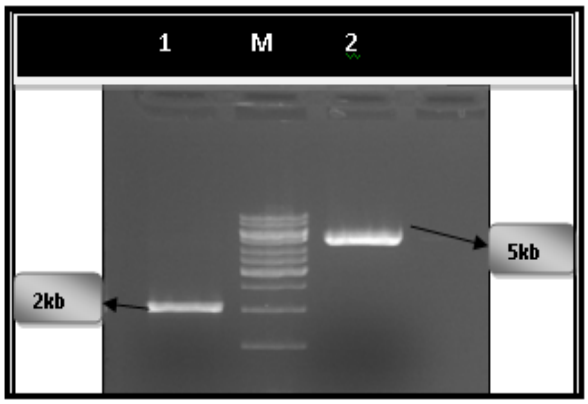

Figure6. Digested product of vector and promoter. M marker, 1: Promoter, 2: pGL3

The digested PCR fragment and pGL3 basic vector were ligated. After overnight incubation at 4°C, producing a circular product that was directly transformed into competent *E.coli DH5α* cells. The transformants were screened on LB-agar plate containing 50 µg/ml ampicillin. Figure7 shows the colonies, which were obtained from transformation after 16 hours incubation at 37°C. These colonies may include the recombinant plasmid carrying the ampicilin resistance gene. To verify the accuracy of transformation, all process including isolation of plasmid from *E. coli DH5α*, digestion with same enzymes was done to get the NQO1gene promoter. Nucleotide sequence analysis of human NQO1 gene promoter revealed that several potential transcription factors binding sites are in the NQO1 promoter. Several transcription factors can bind to these binding sites and Nrf2 is the strongest activator in ARE-mediated NQO1 expression.

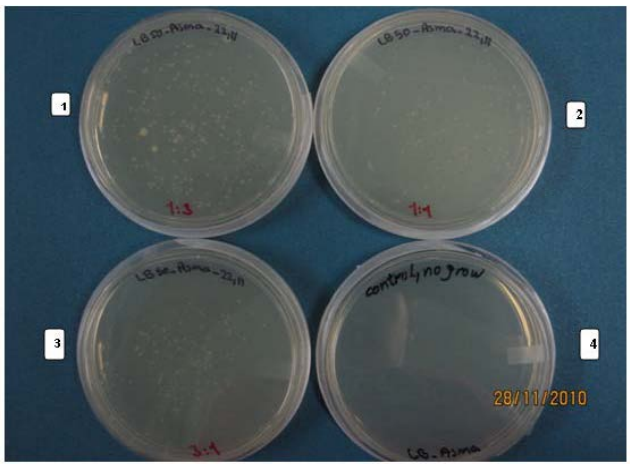

Figure7. Transformation of pGL3-NQO1 promoter into competent E. coli DH5α on LB with 50 µg/mL ampicillin and 4 is the negative control.

4 Conclusion

The sequence of NQO1 gene and promoter were determined with 24230bp and 2123bp. The regulatory sites of NQO1 promoter for binding the transcription factors were characterized using bioinformatics. Primers with restriction cutting sites, which are same with pGL3 Basic vector cutting sites, isolated the promoter from genomic DNA. In this study we successfully isolated the 2123 bp from transcription start site believe to be involve in regulatory the transcription of NQO1 gene. Further investigation proposed potential regulatory regions named ARE located at -477 from the transcription start site. This regulatory region may be responsible in regulating the NQO1 gene expression by polyphenol and further work need to be conduct to validate this.

5 Future Work

Several constructs can be obtained and cloned into pGL3 Basic vector using additional 5` flanking deletion of NQO1 promoter. With these constructs it can be investigated the efficiency of selected polyphenols against the activity of NQO1 gene based on down or up regulation, and possible transcription factors involved in the malaria infected erythrocyte.

6 Acknowledgement

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