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Production of Cyclin D1 Specific siRNAs by Double Strand Processing for Gene Therapy of Esophageal Squamous Cell Carcinoma

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Abstract

Background: (RNA interference) is a new strategy in gene therapy and biotechnology which provides new viewpoints in treatment of different diseases such as cancer and viral diseases. CCND1 which is a key gene in cell cycle is amplified and over expressed in esophageal cancer. The aim of this study was to produce siRNAs for CCND1, the key gene in cell cycle.

Materials and Methods: dsRNA digestion method was applied by using recombinant human dicer enzyme to cleave in vitro transcribed dsRNA into a pool of 22bp siRNA. Total RNA was extracted and cDNA was produced using RT-PCR. T7 promoter was added to both ends of the DNA template by PCR. RNA was produced from both strands of the DNA using T7 RNA polymerase. After annealing both strands, dsRNA was prepared. Finally siRNA pool was produced by dicer treatment.

Results: RNA extraction yield from HN5 cell line was 14.69 µg/10^6 cell. The results from beta actin control gene confirmed the cDNA integrity. After optimization, T7 promoter adding was confirmed using gel electrophoresis and DNA sequencing. After optimization dsRNA yield was improved. The best incubation condition was 18h. Each microgram of dsRNA yielded 0.5 µg siRNA.

Coclusion: dsRNA digestion method includes several steps in which the product of each step is used as the precursor for the next step. So optimization and increasing the specificity and product yield should be the most important goals of the study, Because the yield of each step has a direct relationship with the final product yield namely; siRNA. Optimizing and increasing the yield, dsRNA digestion method could be a rapid, available and profitable method for siRNA generation, and providing large amounts of siRNA.

Keywords: dsRNA digestion; siRNA; RNAi; dsRNA; Esophageal cancer; CCND1

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Introduction

Esophageal cancer is the eighth most common cancer worldwide and the sixth most common cause of death from cancer throughout the world. With variations in its incidence throughout the world, there have been some regions with the highest prevalence rates of esophageal cancer. These regions include the “Asian Esophageal Cancer Belt” from the eastern parts of Turkey to Kazakhstan, Uzbekistan, Tajikistan, Iraq,

Iran and to the western and northern parts of the China, Hong Kong, Japan and the Southern Africa, France and parts of the America (Brazil and Bermuda). The incidence rate of esophageal cancer in the northern parts of China and the shores of the Caspian Sea in Iran is 200 times more than other parts of the world. More than 80% of the morbidity and mortality occur in the developing countries. Esophageal cancer is two to four times more common among men than women. The highest mortality rates are found in both sexes in Eastern and Southern Africa, and in Eastern Asia (1-2). Although the prevalence of different types of esophageal cancer varies in different parts of the world,the most prevalent type is esophageal squamous cell carcinoma (ESCC) throughout the world which is associated with a high mortality rate (2). Carcinogenesis at the cellular level is due to the abnormalities developed in the cell proliferation, differentiation and apoptosis. The genetic alterations observed in ESCC are included: 1) Mutation in P53 tumor suppressor gene, which leads to abnormalities in the cell growth, repair and DNA replication control and apoptosis, 2) Disappearance of the cell cycle control due to the abnormalities in Cyclin-dependent kinase-RB cell cycle control pathway, 3) Oncogene modifications which lead to changes in the signal transduction. There have been many genes involved in these genetic alterations, however, as an oncogene and due to its key role in the cell cycle control which involve tow of the above mentioned mechanisms; Cyclin D1 (CCND1) is one of the most important genes contributed to ESCC pathogenesis (3-7).

Cyclin D1 gene (also known as PRAD1, bcl-1, CCND1) is located on chromosome 11q13 and encodes a 295 amino acid protein which makes complexes with cyclin-dependent kinases and controls a key check point (G1/S) in the cell cycle (8). The amplification and overexpression of the CCND1 gene has been demonstrated in ESCC cell lines (5,8). Therefore,due toits importance, evaluation of CCND1 in ESCC patients is essential and it can be used as a key target in ESCC gene therapy.

RNA interference (RNAi) is one of the new fields in Biopharmaceuticals which makes new point of views in treatment of various diseases. This technology has been used in the treatment of cancer and viral diseases since 2000. Gene silencing as a subgroup of the RNAi technology is mediated with a long (>200nt) double stranded RNA which is digested to 21-23nt dsRNAs with 3'-dinucleotide overhangs (9). In vitro gene-specific silencing has been facilitated with small interfering RNAs (siRNAs) which could be synthesized in the laboratory. siRNAs joint to the complexes called RISC (RNA-induced Silencing Complex), which have an endoribonuclease. Then the sense strand would be dissociated from the antisense and degraded. The antisense attached to the RISC, activates the RISC and directs it to the complement target mRNA which leads to the cleavage of the target mRNA. Therefore the expression of the target gene is silenced in the cell (10-12). Gene silencing using RNAi develops a potent tool for gene function research, drug target identification and the treatmentof diseases (13-18). In addition, siRNA could be used as an effective tool for the elucidation of the cellular pathways.

There have been many methods for siRNA production including: 1) chemical synthesis, 2) enzymatic synthesis (in vitro transcription), 3) long double-stranded RNA digestion or processing using an RNaseIII family enzyme (e.g.dicer),4) endogenous expression as shRNA from expression plasmid or viral vectors, 5) endogenous expression from a PCR-derived siRNA expression cassette, 6) endogenous expression from allosterically regulated ribozymes, 7) using premade siRNAs/shRNAs/lhRNAs (19).

In this study using dsRNA digestion by a human recombinant dicer enzyme, siRNA was produced. This method mimics the natural RNAi process and provides a pool of siRNAs of about 23 base pairs long.

Materials and Methods

siRNA target finding on CCND1 gene

We used the Tuschl method to determine the silencing region. Owing to the existence of exons and introns in the genomic sequence, it was necessary to use the mRNA sequence of the gene which is intron free. The mRNA sequence was extracted from the National Center for Biotechnology Information (NCBI) and according to the siRNA finding instruction, the target sites which were the 19 to 21 nucleotide sequences were determined and surveyed. Thereafter the sequences were analyzed using the BLAST to find the off-targets and after omitting the off-targets, the silencing region on the mRNA was determined.

Total RNA extraction

Total RNA was extracted from HN5 cell line using the RNeasy Mini kit (Qiagen). The integrity and size distribution of the extracted RNA was determined using 1% agarose gel electrophoresis and its concentration was determined using UV spectrophotometer at 260nm. RNA was stored at -70 °C.

cDNA synthesis

First strand cDNA was synthesized using 1µg total RNA, 1µl oligo (dT18) primer (Fermentas) and DEPC-treated water (Applichem) to the final volume of 11.5µl. The mixture was incubated at 60°c (5min)

and after addition of 5x Buffer (Fermentas) (4µl), dNTP (Fermentas) (2µl), RNase inhibitor(Fermentas) (1µl) and Reverse transcriptase (Fermentas) (1µl) it was incubated at 42 °C (60min). In order to stop the reaction, it was incubated at 70 °C (10min). cDNA was stored at -20 °C. In order to confirm the cDNA synthesis, polymerase chain reaction (PCR) was performed using a control gene. Beta action was selected as the control gene using forward (5'-ATGGCCACGGCTGCTTCCAGC3') and reverse (5'CAGGAGGAGCAATGATCTTGA3') primers. The reaction was consisted of 2.5µl 10x PCR buffer (Bioron), 1µl forward and reverse primers, 2µl MgCl2 (25mM) (Bioron), 2µl cDNA and ddH2O to the final volume of 25µl. After an initial heating step (95 °C for 2min), the thermal program was 95 °C 30 sec, 60.8 °C 30 sec and 72 °C 30 sec, for 35 cycles. The reaction mixture was heated at 72 °C for 5min and stored at 4 °C. The amplified beta actin gene was visualized using 1% agarose gel electrophoresis.

Amplification of the target silencing region of CCND1 gene using PCR

The reaction mixture was consisted of 12.5µl 2x PCR buffer (Fermentas), 1µl forward (5'CTGTGCTG-CGAAGTGGAAAC3') and reverse (5'TGAGGCG-GTAGTAGGACAGG3') CCND1 primers, 2µl cDNA and ddH2O to the final volume of 25µl. After an initial heating step (94 °C for 3min), the thermal program was 94 °C 30 sec, 56.3 °C 30 sec and 72 °C 1.5min, for 35 cycles. The reaction mixture was heated at 72 °C for 10min and stored at 4°c. The amplified target silencing region of CCND1 gene which was 750 bp, was visualized using 1% agarose gel electrophoresis.

T7-promotor addition using PCR

The reaction mixture was consisted of 12.5µl 2x PCR buffer (Fermentas), 1µl forward (5'GCGTAATAC-GACTCACTATAGGGAGACTGTGCTGCGAAGT GGAAAC3') and reverse (5'GCGTAATACGA-CTCACTATAGGGAGATGAGGCGGTAGTAGGA CAGG3') CCND1 (with T7-promotor) primers, 2µl DNA and ddH2O to the final volume of 25µl. After an initial heating step (94 °C for 3min), the thermal program was 94 °C 30 sec, 61.4 °c 30 sec and 72 °C 1.5min, for 35 cycles. The reaction mixture was heated at 72 °C for 10 min and stored at 4 °C. The amplified target silencing region of CCND1 gene with the added T7-promotor which was 790 bp, was visualized using 1% agarose gel electrophoresis.

Double-stranded RNA (dsRNA) production (transcription)

The reaction mixture was consisted of 10µl 10x transcription buffer (Fermentas), 6µl NTP (Fermentas), 50U RNase inhibitor (Fermentas), 30U T7 RNA polymerase (Fermentas), 2µg DNA and DEPC-treated water (Applichem) to the final volume of 50µl. After incubation at 37 °C for 18hr, 2µl DNase (Fermentas) was added to the mixture and it was incubated at 37 °C for 15min, 70 °C for 10min and room temperature for 20min. dsRNA was visualized on 1% agarose gel electrophoresis.

dsRNA digestion and siRNA production

The reaction mixture was consisted of 1µg dsRNA, 1µl ATP (10mM) (GenLantis), 0.5µl MgCl2 (50mM) (GenLantis), 4µl Dicer reaction buffer (GenLantis), 2µl recombinant dicer enzyme (GenLantis) and RNase-free water to the final volume of 10µl. After incubation at 37 °C for 18hr, 2µl Dicer Stop Solution (GenLantis) was added to the mixture. The produced siRNAs with about 22bp length were visualized on 1% agarose gel electrophoresis. siRNA purification was performed using Dicer kit (GenLantis) according to the manufacturer's protocol.

Results

siRNA target finding on CCND1 gene

CCND1 gene sequence (Gene ID: 595) was extracted from the NCBI. According to the Tuschl protocol for siRNA target finding resulted in 10 target sites for siRNA function in the region of 210 to 1097 on the mRNA sequence of CCND1 gene. The best sequence candidates were located at region 700 to 750 of the sequence. In homology searches, this region had no homology to the other genes.

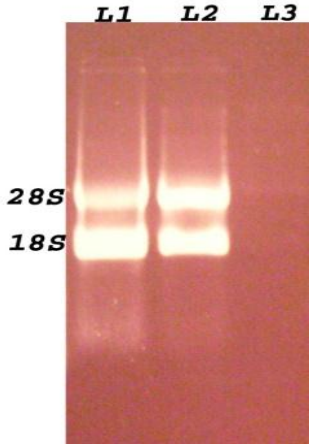

Figure 1. Total RNA extraction from HN5 cell line. 18s and 28s bands could be seen.

Total RNA extraction

Total RNA concentration was 847ng/µl and the

extraction yield was about 14.69µg RNA per million cells. The integrity and quality of the extracted RNA was checked using gel electrophoresis (Figure 1).

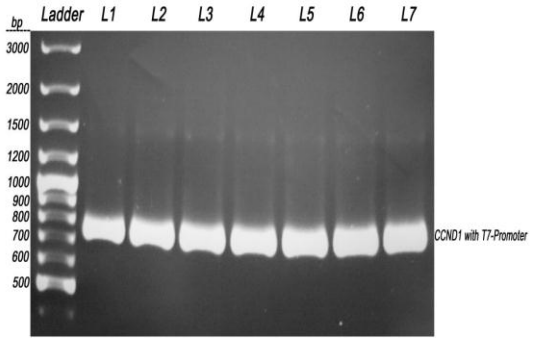

Figure 2. Gel electrophoresis of PCR performed on cDNA using beta actin and CCND1 primers. Beta actin selected as the control gene.

cDNA synthesis and Amplification of the target silencing region of CCND1 gene using PCR

The amplified beta actin gene was visualized using 1% agarose gel electrophoresis (Figure 2). The amplified target silencing region of CCND1 gene which was 750 bp, was visualized using 1% agarose gel electrophoresis (Figure 2).

T7-promotor addition using PCR

T7-promotor addition to the target silencing region of CCND1 gene was performed by addition of 38 bases of the T7-promotor to the 21bp CCND1 primer. The amplified target silencing region of CCND1 gene with the added T7-promotor which was 790 bp,was visualized using 1% agarose gel electrophoresis (Figure 3).

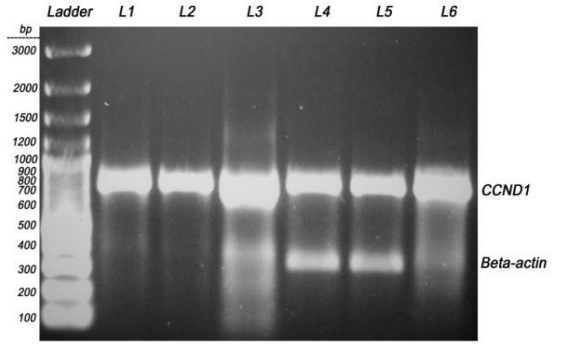

Figure 3. Gel electrophoresis of PCR performed to add the T7-promotor to the target silencing region of CCND1 gene

Double-stranded RNA (dsRNA) production (transcription)

dsRNA with 790bp lengthg was visualized on 1% agarose gel electrophoresis (Figure 4). dsRNA concentration was 40µg/µl.

Double-stranded RNA (dsRNA) production (transcription)

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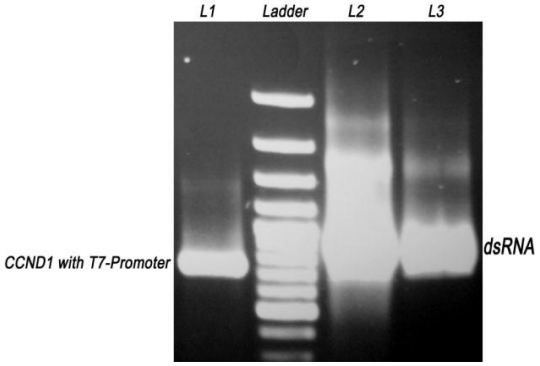

Figure 1. Gel electrophoresis of the produced dsRNA

dsRNA digestion and siRNA production

The produced siRNAs with about 22bp length were visualized on 1% agarose gel electrophoresis (Figure 5).

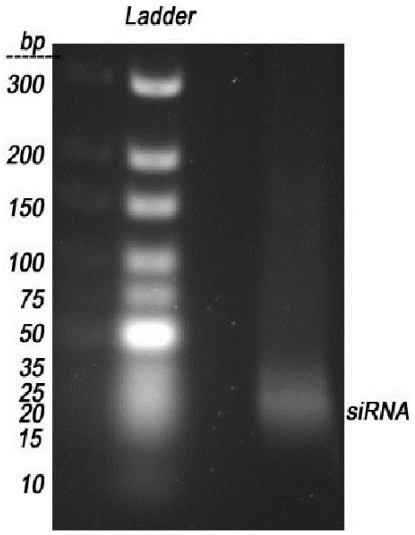

Figure 2. Gel electrophoresis of the produced siRNA after enzymatic dsRNA digestion

Discussion

As a new field in gene silencing technology, RNAi is expected to revolutionize the genetic and molecular researches in future which would have a drastic effect on the treatment of diseases. Overexpression of genes in cancers, autoimmune and other diseases have an important effect on these diseases and even sometimes is the main cause of the disease; accordingly processes which could specifically revise the incorrect gene expression without disturbing other

genes are very worthwhile. Therefore siRNA is expected to be the new generation drugs in the future.
The main objective of this study was siRNA production in the laboratory and accommodation of the technology for further uses. Since siRNA is a dsRNA with target specificity which has no definite sequence, it has diverse structures and therefore it should be synthesized specifically for each target which makes its production technology so valuable (14).
siRNA production methods in the laboratory are divided into three main groups: 1) in vitro production using vectors, 2) chemical synthesis which needs special equipments and is usually done with companies, 3) enzymatic production using T7 RNA polymerase (19). The vector method has two main drawbacks including transient expression in the cells and its formulation. Although rapid and accurate, chemical synthesis is very expensive for research centers and therefore the technology could never be used in the laboratories. Large scale production using this method is very expensive and could not be available in large amounts, while large amounts of siRNA in needed for the formulation studies of siRNA. According to its low cost, production of pure siRNA without the vector disturbance and knowledge about the technology of production, in this study the enzymatic synthesis of siRNA using T7 RNA polymerase enzyme was selected as the best method for siRNA production. This method includes a PCR, a transcription in the laboratory and an enzymatic digestion in the laboratory (19).
siRNA target region finding is the key step in the siRNA production method. This region should be analyzed using BLAST and the unique sequence should be found. The CCND1 sequence has low sequences specific to the cyclin genes which have been used in this study for specific siRNA designing.
In the enzymatic synthesis method for siRNA production, the yield in each step affects drastically on the yield of the last step which using optimization and qualification of the product and, we tried to increase the final yield. The main problem in the T7-promotor addition to the target region was the primer dimmer formation due toue to length of the primer used. In the dsRNA synthesis using 1µg template, 9µg dsRNA was produced. Since the two strands of RNA are complement, there has been the possibility that the hybridization occurred improperly which it is better to perform this process in the laboratory again.
Dicer is similar to the RNase III enzyme in prokaryotes and its effect on the dsRNA leads to 22bp siRNAs. In this study the efficiency of the produced siRNAs has been analyzed in other project.
The study performed in 2002 by Picard and Donze which was similar to our study, used the enzymatic production method and resulted in 80% inhibition using western blotting method. In the study done by Donze, one siRNA type was produced but in our study a target region with 10 siRNA sites was selected and T7-promotor was added which finally resulted in a pool of siRNAs.
In gene silencing using siRNA it should be considered that according to the mRNA structure in the cytoplasm ,it is possible that an siRNA target site would not be available which leads to a decrease in siRNA efficiency, Therefore when using one type of siRNA in a research, it is possible that the maximum effect of the gene silencing could not be seen, while with a pool of siRNAs the maximum effect could be seen which in the following studies the specific siRNA could be selected and produced and formulated.
Briefly, pure siRNA production in large scale is valuable which facilitates the research and drug formulation for the clinical trials.

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