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siRNA KOCKDOWN OF TrpRS INDUCES APOPTOSIS, INHIBITS CELL PROLIFERATION AND ARRESTS CELL GROWTH IN HUMAN CERVICAL CANCER CELLS

JOYEETA SENGUPTA[a], CHENGFU YUAN[b], XIANGMEI WU[c], HUAN LAN[d], YOUQUAN BU[e], FA PING YI[f] AND FANG ZHOU SONG[g1]

Molecular Medicine and Cancer Research Center, Department of Biochemistry and Molecular Biology, Chongqing Medical University, Chongqing, PR China

[a] E-mail: lotus.cqmu@gmail.com
[b] E-mail: yuancf46@163.com
[c] E-mail: xmwu80@163.com
[d] E-mail: lh6402196@126.com
[e] E-mail: buyqcn@yahoo.com.cn
[f] E-mail: fazicq@163.com
[g] E-mail: fzsongcq@163.com

ABSTRACT

TrpRS or Tyrptophanyl-tRNA synthetase is among the first proteins that appeared in evolution. The purpose of the present study is to evaluate the effect of silencing of TrpRS gene by RNA interference in human cervical cancer cells. Small interfering RNA (siRNA) was designed to knock down the expression of TrpRS gene. The effect of knockdown was studied by real time PCR and western blot. The effect of TrpRS silencing on cell proliferation was studied by CCK8 kit. Effect of silencing on apoptosis and cell cycle was studied by FACS. Real time PCR was performed to study the expression of BAX as well as the behavioral pattern of TrpRS in the cell cycle. The results revealed that siRNA knockdown of TrpRS caused a gradual decline in the proliferation of cervix cancer cells, induced apoptosis, increased the expression of BAX gene, caused accumulation of cells in the G1 phase of cell cycle, also inhibited cell transition from G1 to S phase, in addition to inhibiting the expression of cyclin E & cdk2. These results suggest that, siRNA knockdown of TrpRS gene on human cervix cancer cells may be subjected to rigorous study with a view to develop as gene therapy in patients with cervical cancer.

KEY WORDS: TrpRS, cervix cancer, siRNA, gene therapy

Cancer of the cervix today has been one of the commonest causes of cancer in women worldwide. Uterine cervical cancer is still the leading cause of cancer deaths for women in many of the developing countries (Greenlee et al., 2000 and Parkin et al., 1999), including India and China. Owing to lack of effective screening programs for cervical cancer in developing countries, no clinically significant reduction in the incidence of cervical cancer has occurred during the past three decades (Rengaswamy et al., 2009). Whereas in developed countries, a major decline in cervical-cancer mortality has been achieved after the introduction of large-scale cytologic testing (Rengaswamy et al., 2009).

Silencing of gene for gene therapy is a novel technique used these days to target and turnoff single gene in a cell (McMillan; 2005).The first step of protein synthesis that consists of the aminoacylation of tRNAs, is catalyzed by aminoacyl-tRNA synthetases. Other than protein synthesis, aminoacyl-tRNA includes function like transcriptional and translational regulation and cell signaling (Zeng et al., 2008).The ligation of amino acids to their cognate tRNAs is catalysed by aminoacyl tRNA transferase which is essential for protein synthesis and cell viability. The basic reaction is:

AA + ATP +tRNA→AA- tRNA + AMP + PP. The reactions require the capacity to recognize tRNAs as well as small chemicals such as amino acids and ATP. Hence, the structures of AARSs are well equipped for interacting with diverse molecules that may be associated with their functional versatility. Thus, the catalytic activities for glycyl-, lysyl-, and tryptophanyl-tRNA synthetase have been adapted to synthesize diadenosine polyphosphates (Ap_nA), which are believed to regulate glucose metabolism cell proliferation, and death(Park et al., 2008).

TrpRS is believed to be among the first proteins evolved during evolution. Two forms of Tryptophanyl-tRNA synthetase exist, a cytoplasmic form, named WARS, and a mitochondrial form, named WARS2. Tryptophanyl-tRNA synthetase belongs to the class I tRNA synthetase family. Four transcript variants encoding two different

[1]**Corresponding author**

isoforms have been found for this gene. Cross species aminoacylation is an effective phylogenetic probe to learn the close relation between eukarya and archaea. Recognition process of TrpRS-$tRNA^{TRP}$ of a G73/A73 dichotomy, divides the bacterial and archaeal eukaryotic phylogenetic domains (Guo et al., 2002)

Normally in a cell, TrpRS exists as full-length protein and as a truncated TrpRS (mini-TrpRS) in which most of the extra NH_2-terminal domain is deleted due to alternative splicing of the pre-mRNA (Kise et al., 2004; Jorgensen et al., 2000) with Met-48 which is deduced as NH_2-terminal residue of mini-TrpRS (Kise et al., 2004). Human TrpRS was shown to split into two fragments and possess novel cytokine functions. The carboxy terminal domain manifested chemotactic activity while the amino terminal domain could bind to interleukininterleukin-8 type. A full length enzyme is secreted in cell culture under apoptotic condition which may contribute to apoptosis (Wakasugi and Schimmel, 1999). TrpRS has been reported to have opposing activity with TyrRS on endothelial cell angiogenesis in the matrigel assays. It has been suggested that the opposing activities of the two tRNA synthetases hints at the tight regulation of the balance between pro- and anti-angiogenic stimuli (Zeng et al., 2008). Although TrpRS has been subjected to study in last two decades, no study till date has been reported on human cervical cancer. As gene silencing is thought to be one of the novel technique to target a single gene, we targeted to knockdown the expression of TrpRS by RNAi interference and study the effect of silencing on the behavioral pattern of TrpRS in human cervical cancer cell lines-HeLa, SiHa and CaSki cells. Precisely, the results obtained in this study suggest that TrpRS could be studied further for its use in gene therapy in context to cervix cancer.

MATERIALS AND METHODS

Cell Culture

HeLa, SiHa and CaSki cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were incubated in an incubator with an atmosphere of 5% CO_2 at 37 °C. Cells were transfected using Lipofectamine RNAiMAX (Invitrogen/Life Technologies, Carlsbad, CA, USA). Six well format was maintained during transfection and manufacturer's instruction was followed. A number of $1x10^5$ cells per well was maintained during transfection.

Designing of siRNA Target Site

The siRNA was designed and purchased from Gene pharma (Shanghai, China) as per the following protocol. Keeping the AUG start codon, the transcript was scanned with AA dinucleotide sequences. Three siRNA target sites at different positions along the length of the gene sequence were selected.

Each AA and the 3′ adjacent 19 nucleotides were considered as potential siRNA target sites. The ratio G/C ratio was 30%-50 % The G/C ratio was measured with Ambion siRNA analysis software. (http://www.ambion.com/techlib/misc/siRNA_finder.html).

Correlation between the position of target sites on the mRNA and siRNA potency was not evaluated. The potential target sites was compared to the appropriate genome database and any target sequences with more than 1617 contiguous base pairs of homology to other coding sequences was avoided. BLAST was used from the NCBI server at: http://www.ncbi.nlm.nih.gov/BLAST. Negative control siRNA was constructed with the same nucleotide composition as siRNA but lacking significant sequence homology to the genome.

siRNA Template Oligonucleotide Design, Preparation and Selection

The three oligonucleotide designed are as following:

WARS-homo-924: top strand 5'GGCUCCAGGAU GUAUUUAATT 3', and bottom strand 5'UUAAAUACAU CCUGGAGCCTT3'; WARS-homo-2844: top strand 5'GUCACCCAGUAGAGUAAAUTT3', bottom strand 5'AUUUACUCUACUGGGUGACTT3'; WARS-homo-1108: top strand 5'GAGCUCAGGUUCUACAAATT3', bottom strand 5'AUUUACUCUACUGGGUGACTT3'. All the three designed target siRNA and the negative controls were diluted with DEPC to make a final concentration of 20µM as per manufacturer's instruction. All the three siRNAs were transfected to HeLa cells to choose the best target site using Lipofectamine RNAiMAX purchased from

Invitrogen. Transfection was done as per the 6 well format protocol provided by the Invitrogen. A day prior to transfection, cells were plated in 2 ml of growth medium without antibiotics such that they were 30-50% confluent at the time of transfection. A number of $1x10^5$ cells per well was maintained during transfection. To obtain the highest transfection and low non-specific effects, the RNA and Lipofectamine™ RNAiMAX concentrations were varied. 0.5ml siRNA oligomer-Lipofectamine™ RNAiMAX complexes were added to make the final volume of 2.5 ml in each well. The cells were incubated at 37ºCfor 48 hrs.

Real Time Fluorescent Quantitation PCR (RT-PCR)

After transfection the total RNA was isolated from HeLa cell using the Trizol reagent (MRC, USA) according to the manufacturer's instructions. 5 µL of total RNA was converted to complementary DNA (cDNA) with Revert Aid™ First Strand cDNA Synthesis Kit. An aliquot of 1µL of the resulting cDNA was used as template for PCR amplification with the following primers: human TrpRS, P1 (forward, 5′-CCC TGC TGC ACT CCA CCT T-3′), P2 (reverse, 5′-ACG CAT GCT TAT TGA CCT TG-3′) (Zeng et al., 2008). The PCR condition maintained is as follows -an initial denaturation (94 °C for 2 min), followed by 45 cycles of denaturation, annealing and extension (94 °C for 20 s, 54 °C for 20 s, 72 °C for 30 s), and a final extension (72 °C for 5 min) Fig (1a). The transcript of GAPDH was also amplified by RT-PCR from the same cDNA template and was used as an internal control and was synthesized by Takara (Dalian, China). The silencing efficiency was also checked by semi-quantitative PCR and the bands were scanned and analyzed by the Gel Doc 1000 gel imaging system Fig (1b). The PCR conditions maintained were same as that of Real time PCR as mentioned above.

Western Blot Analysis

Cytoplasmic protein extracts were prepared according to the instructions of the BCA protein extraction kit purchased from Pierce (Rockford, IL, USA). Rabbit polyclonal anti-human TrpRS IgG antibodies and horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG were purchased from Abcam (Hong Kong). Protein marker was purchased from Fermentas (Shenzhen, China) .The ECL chemiluminescence kit was purchased from Pierce (Rockford, IL, USA). The extracted proteins (30 µg) were subjected to 10% SDS-PAGE. After electrophoresis, proteins were transferred onto PVDF membranes (Millipore, Billerica, MA, USA). The membranes were then blocked using blocking buffer containing nonfat dry milk. Rabbit polyclonal anti-human TrpRS IgG antibodies (final dilution 1:5000) were used as the primary antibody, and horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (final dilution 1:10000) was used as the secondary reagent. An internal control, HRP-conjugated beta actin and Protein Marker were also used in this experiment. Detection was performed using an ECL chemiluminescence kit. The data were scanned and analyzed by the Gel Doc 1000 gel imaging system (Bio-Rad Company, Hercules, CA, USA). The experiment was repeated 3 times.

Apoptosis Analysis

The effect of silencing of gene on apoptosis was checked on HeLa, SiHa and CaSki as follows: a day prior to transfection, cells were plated in 2 ml of growth medium without antibiotics such that they were 30-50% confluent at the time of transfection. A number of $1x10^5$ cells per well was maintained during transfection. Cells were treated with or without siRNA. Lipofectamine RNAiMAX (Invitrogen/Life Technologies, Carlsbad, CA, USA) was used to transfect the cells.The 6 well format was followed as per manufacturer's protocol. An Annexin V-FITC apoptosis detection kit (BD Pharmingen) was used to detect early apoptotic activity according to the manufacturer's instructions, with slight modifications. The cells were harvested, washed twice with ice-cold PBS, and resuspended in binding buffer. Annexin V-FITC and propidium iodide (PI) were then added and incubated for 15 min at room temperature in the dark. Cells were finally analyzed for apoptosis by flow cytometry.The experiment was repeated three times on all three cervix cancer cell lines. Further the effect of silencing on expression of BAX was studied by real time PCR.

Cell Proliferation Assay

Effects of RNAi interference of TrpRS on proliferation of cells was done with cell counting kit-8 (Dojindo) for HeLa, SiHa and CaSki cells as per 96 well format. In brief, a cell suspension of 50µl of growth medium without antibiotics was made the previous night before transfection such that they were 30-50% confluent at the time of transfection. Next day the cells were treated with or

without siRNA. A set of four 96well plate were prepared for HeLa, SiHa and CaSki cell line each to study at 0h,24h, 48h and 72h. For every plate 10µl of cck8 was added and incubated for 2hrs at 37º C prior to study their absorbance. Absorbance was studied by microplate reader at 450nm.The experiment was repeated three times on each cell line.

Cell Cycle Analysis

The effect of silencing of gene on cell cycle of all three cell lines was checked as per follows. A day before transfection, cells were plated in 2 ml of growth medium without antibiotics such that they were 30-50% confluent at the time of transfection. A number of $1x10^5$ cells per well was maintained during transfection. Cells were treated with or without siRNA. Lipofectamine RNAiMAX (Invitrogen/Life Technologies, Carlsbad, CA, USA) was used to transfect the cells. The 6 well format was followed as per manufacturer's protocol.48 hrs after transfection, the cells were collected by trypsinisation, washed with cold PBS, and fixed in ethanol at 4°C for at least 4 h. The fixed cells were collected by brief centrifugation and resuspended in PBS containing 0.1% BSA and 0.01% NaN3. The cells were then treated with RNase A and stained with propidium iodide (PI) for 15 min at room temperature. The samples were finally subjected to cell cycle analysis on a FAC scan flow cytometer. The experiment was repeated three times. Real time PCR was performed to check the effect of silencing on the expression of cyclin E and cdk2 to analyse the behavior of TrpRS in the G1-S transition in the cell cycle.

Statistical Analysis

The values after statistical analysis were represented as mean±SD. Paired t-test was performed to compare between means of two samples using Microsoft excel 2007 version. $p<0.05$ was considered to be statistically significant.

RESULTS

siRNA that Exhibited Maximum Knockdown of TrpRS in HeLa cells

All the three designed siRNA successfully reduced the expression of TrpRS. Out of the three designed siRNA, WARS-homo-1108 showed maximum efficiency in knocking down the expression of TrpRS in HeLa cells. Thus, WARS-homo-1108 was chosen for further analytic experiment on HeLa, SiHa and CaSki cells fig. 1(a) & 1(b). GAPDH was used as positive control.

TrpRS Knockdown Inhibited Protein Formation in HeLa cells

Western blot analysis showed that the silencing of TrpRS influenced the mRNA resulting in reduced quantity of protein formation as evident in fig. 2. The density of the band after silencing was much reduced, compared to that of cells without siRNA as well as negative control. So, as to investigate the effect of TrpRS knockdown by siRNA on different parameters, further experiments were conducted on HeLa, SiHa and CaSki cells.

RNAi mediatedTrpRS Silencing Inhibits Cell Proliferation in HeLa, SiHa and CaSki cells

A decrease in the number of siRNA transfected cells compared to that of untransfected and negative control was noticed as shown in fig. 4. In HeLa and SiHa cells ,a significant decrease in the proliferation of cells has been noticed after 48h and 72h after TrpRS silencing as compared to the negative control as well as untreated cells but no significant difference was noticed between 0h and 24h post transfection ($p<0.05$). In CaSki cells, a significant decrease in cell proliferation was noticed at 24h, 48h and 72h after transfection as compared to that of negative and untreated cells ($p<0.05$).

RNAi mediated TrpRS Silencing Induced Apoptosis in HeLa, SiHa and CaSki cells

RNAi mediated silencing of TrpRS showed higher induction of apoptosis in all the three cervix cancer cell lines as seen in fig. 3(a).The increase in apoptosis level was found significant ($p<0.05$) as compared to their negative control as well untreated cells, in HeLa, SiHa and CaSki cells. An increase in the expression of BAX gene was notice after silencing of TrpRS as compared to that of untreated as well as negative control cells, fig .3(b).

RNAi mediated TrpRS Silencing Affects the Transition of Cells from G1 Phase to S Phase of the Cell Cycle in HeLa, SiHa and CaSki Cells

A reduced number of cell transition from G1 to S phase in HeLa, SiHa and CaSki cells was seen as shown in fig 5 (a).The decrease in number of cells were found to be significant as compared to the negative control as well as untransfected cells ($p<0.05$). Real time PCR analysis of

cyclin E/cdk 2 showed a decrease in the expression of both cyclin E and cdk2 after siRNA silencing of TrpRS, fig. 5(b).

DISCUSSION

RNA interference by double stranded RNA (dsRNAs) molecules of approximately 20-25 nucleotides termed short interfering (siRNAs) is a powerful method for preventing the expression of a particular gene. The dsRNA molecules can use cellular process to target mRNAs with complementary sequence for degradation (Elbashir et al., 2001).This technique was first developed in *Caenorhabditis elegans*, and was then applied to a wide range of organisms at large. Usage of RNAi is proving to be an invaluable research tool that allows rapid characterization of the function of known genes. More importantly, RNAi technology supports identification of novel genes involved in disease processes (Mocellin and Provenzano, 2004). In normal cells, human TrpRS exists as a full length form and as a truncated form designated mini-TrpRS, which is produced by alternative splicing (Tzima et al., 2005). siRNA chemically synthesized or in vitro transcribed can be transfected into cells, injected into mice, or introduced into plants (Brummelkamp et al., 2002).TrpRS is thought to be closely related to protein biosynthesis and angiogenesis (Yang et al., 2004). siRNA has been used to knockdown mini TrpRS in human umbilical vein endothelial cells to study the angiostatic activity of mini-TrpRS (Zeng et al., 2008). Similarly angiostatic activity of mini TrpRS on myocardial infarction in rats has been successfully studied by siRNA knockdown of TrpRS (Zeng et al., 2010). A fragment of human TrpRS can be used as antiangiogenic for ocular angiogenesis (Otani et al., 2002).No previous study of TrpRS on cervix cancer cells has been reported till date.

In this present study, we successfully knocked down the expression of TrpRS by siRNA oligonucleotide in HeLa cell. The first finding, in which siRNA significantly decreased the proliferation of cells in HeLa, SiHa and CaSki cells could be related to the findings obtained from apoptosis and cell cycle analysis. An increase in the expression of BAX gene and increase in the rate of apoptosis was noticed after RNAi mediated silencing of TrpRS gene. Both these findings suggest that probably BAX gene, which is a proapoptotic member of Bcl-2 protein family, has been triggered by siRNA silencing of TrpRS. When Bcl-2 over expresses, Bcl-2-Bax heterogenous dimer predominates, resulting in inhibition of apoptosis (Oltvai et al., 1993) whereas, when Bax expresses excessively, Bax-Bax homogenous dimer or monomer predominates, resulting in apoptosis (Wu et al., 2011) This is consistent with our finding of apoptosis in this study. Probably silencing of TrpRS resulted in predomination of Bax-Bax homogenous dimer or monomer leading to an over expression of BAX gene causing increased apoptosis of the cervix cancer cells.

A proliferating cell consists of four stages in the cell cycle G1, S, G2 and M phase. G1 is characterized by cell growth followed by proliferation; S phase consists of DNA replication which is followed by G2 during which cells prepares itself for growth and division. Passage through the four phases in the cell cycle is controlled by cyclin dependent Kinases (cdks). The cyclin/cdk complex is regulated by phosphorylation and dephosphorylation of the key residues of the complex which are located on the cdk subunits. (Nakayama et al., 2001;Bartek and Lucas, 200; Harbour and Dean, 2000 and Momand et al., 2000).

In our study, cell cycle analysis showed a decrease in the cell content in S phase as well as decrease in expression of cyclin E and cdk 2 after silencing of TrpRS. These finding hints at the possibility of TrpRS affecting the cyclin E/cdk2 complex required for transition from G1 to S phase. Further, inhibition in the expression of cyclin E and cdk after silencing suggests that silencing of TrpRS may have inhibited the binding of cyclin E-cdk2 or altered the cyclin/cdk complex in a certain way which led to reduced number of cell transition to S phase. To confirm the above mentioned assumption and study the exact mechanism of action of TrpRS in the G1-S transition, a detailed study is required.

Considering the above mentioned results, we can be assume the followings. Firstly, siRNA silencing of TrpRS increased the expression of BAX gene which may have induced apoptosis. Secondly, silencing of TrpRS may has decreased the cell transition from G1-S phase by inhibiting cyclin E-cdk2 complex. So, both of these might have caused the inhibition in cell proliferation as noticed in HeLa, SiHa and CaSki cells. A detailed study is awaited to explore more

of the behavioral pattern of TrpRS. Induction of apoptosis and inhibition in the cell proliferation altogether is a good behavioral manifestation in context to therapy development for cancer cells. For now, we report our results obtained from the study on silencing of TrpRS gene by RNAi interference on cervix cancer cells and suggest that TrpRS could be a good candidate for subjecting to study in context to gene therapy in human cervical cancer cells.

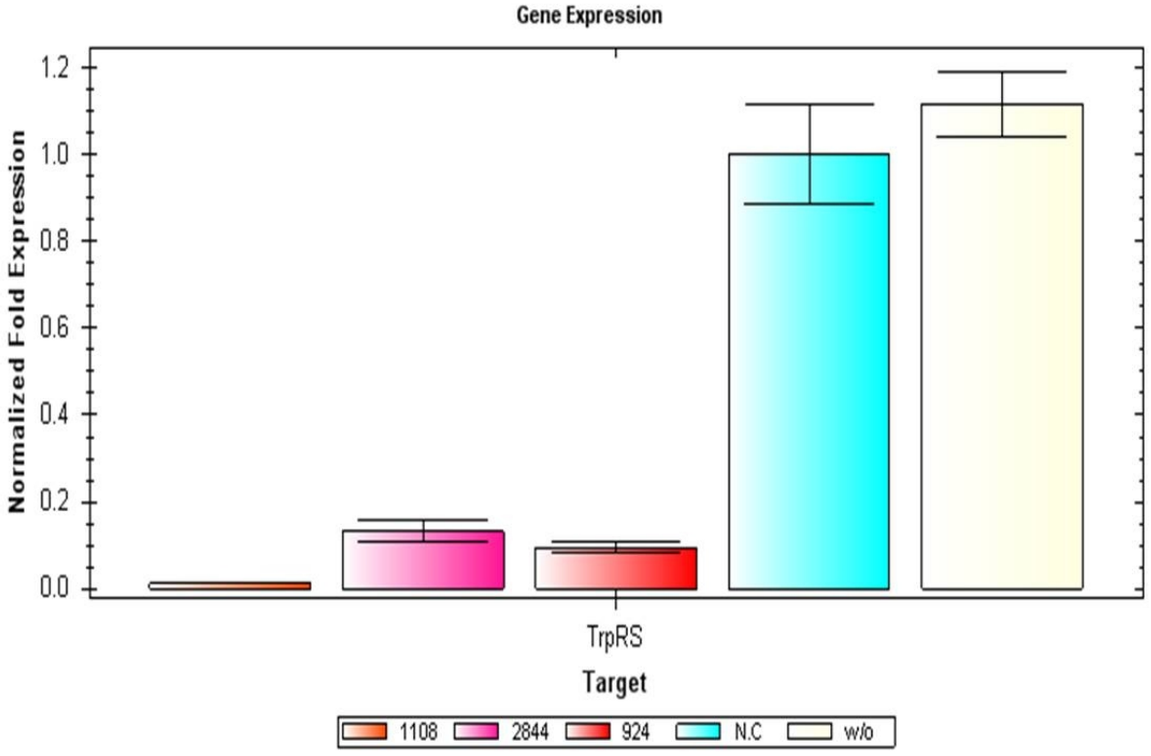

Fig. 1a: Showing real time PCR analysis of silencing of TrpRS by three different small interfering RNA in HeLa cells. Out ofthree designed siRNAs, WARS-homo-1108 showed highest knockdown of TrpRS. W/O are untreated cells and N.C is negative control

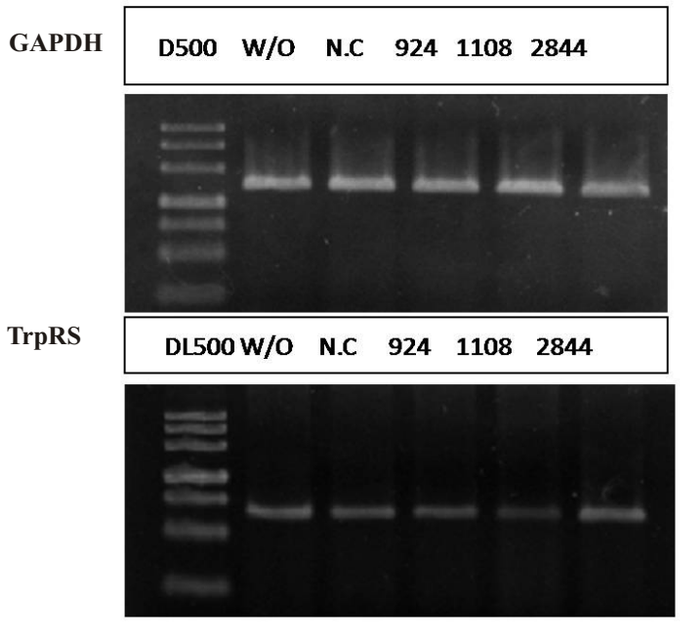

Fig. 1b: Showing that WARS-homo-1108 knockdowns the expression of TrpRS gene maximum in HeLa cells. This is consistents with the real time PCR analysis as shown in Fig. 1(a). GAPDH was used as positive control. W/O are untreated cells and N.C is negative control. WARS-homo-1108 was chosen to carry out further experiments

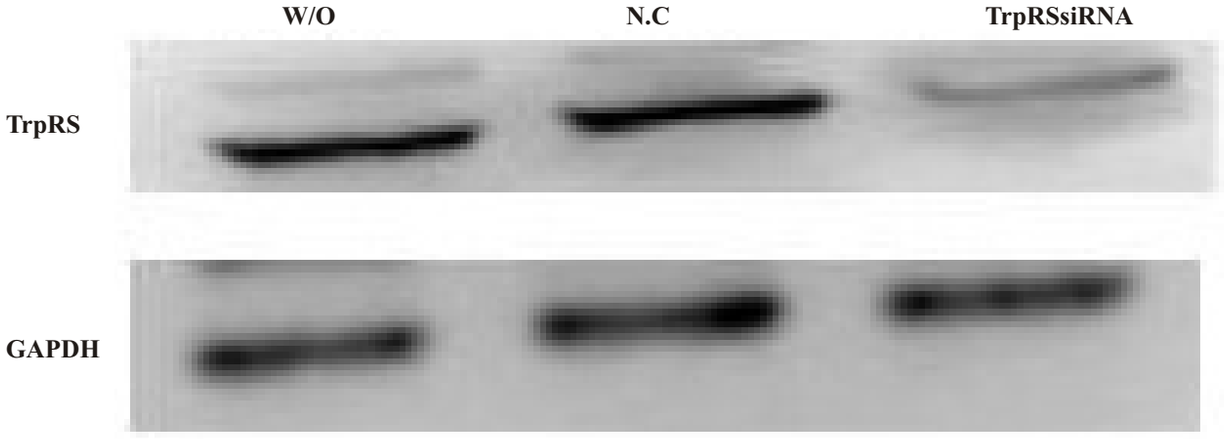

Fig .2 : Showing effect of silencing of TrpRS on protein formation in HeLa cells. Decrease in the expression of protein after TrpRS silencing by RNA interface has been noticed as compared to the negative and untransfected cells. GAPDH was used as positive control. W/O are untreated HeLa cells and N.C is negative control

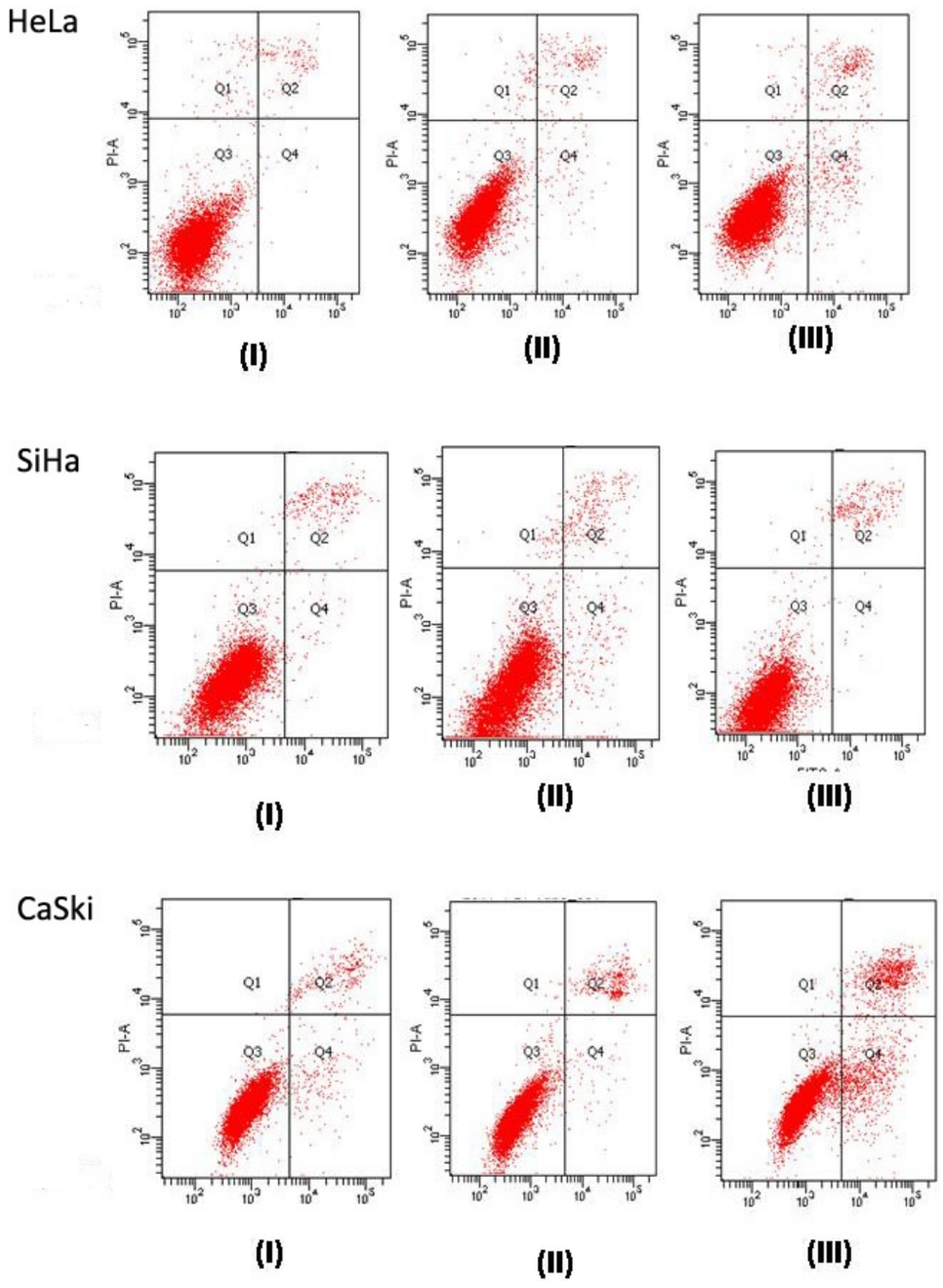

Fig. 3 a: Showing the effect of silencing of TrpRS on apoptosis in HeLa, SiHa and CaSki cells, where (I) represent cells without SiRNA treatment, (II) represents cells transfected with negative control SiRNA and (III) represents cells transfected with SiRNA targeting TrpRs. Apoptosis observed for I, II and III in HeLa cells are 3, 1.6 and 5.8%, in SiHa cells are 2.5, 2.2 and 4.7%, in CaSki cells are 4.6, 4.2 and 15.8%. Induction of apoptosis has been seen after SiRNA silencing of TrpRS. The difference in the values of apoptosis were found to be significant ($p<0.05$)

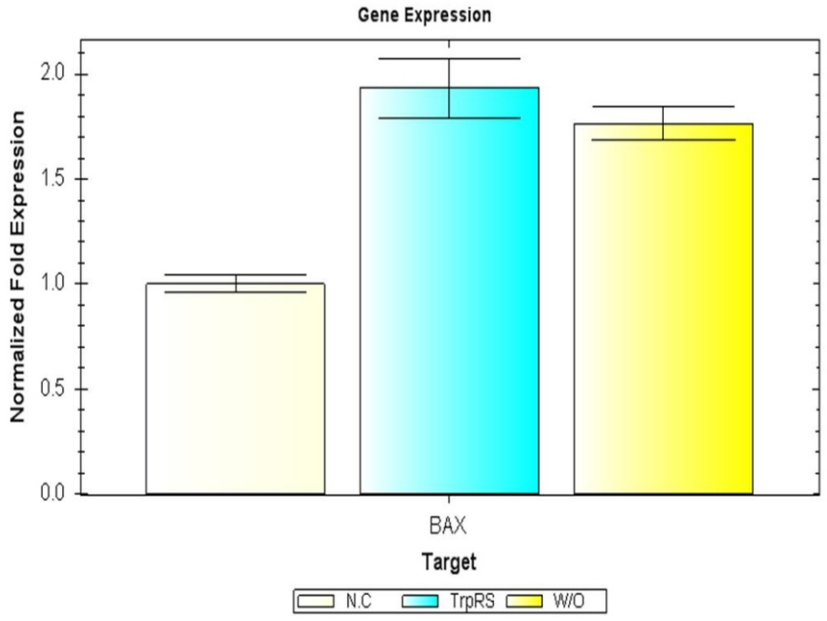

Fig.3b: Showing the effect of silencing of TrpRS on expression of Bax gene in HeLa cells. The expression of Bax gene was enhanced after silencing compared to that of the negative control as well as untreated cells causing induction of apoptosis in HeLa, SiHa and CaSki cells post transfection

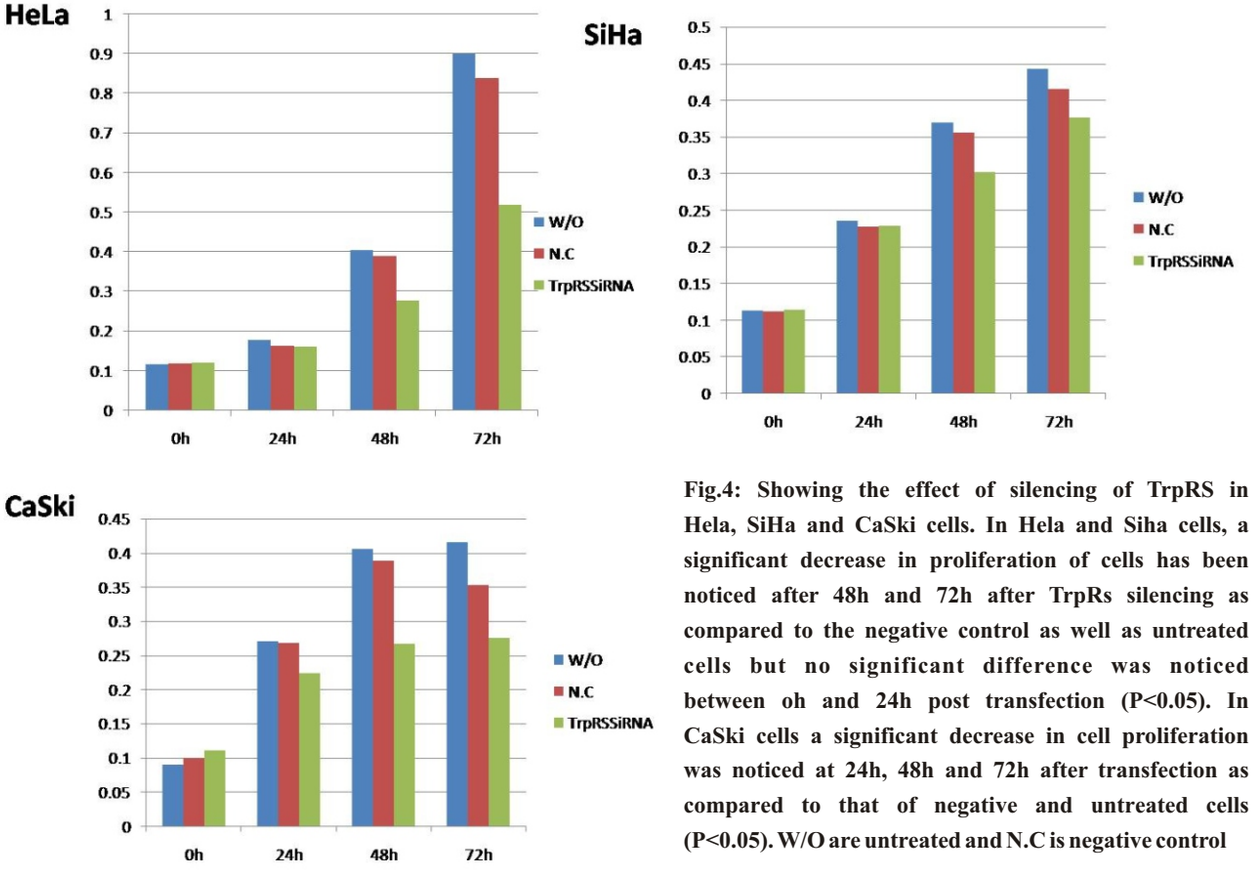

Fig.4: Showing the effect of silencing of TrpRS in Hela, SiHa and CaSki cells. In Hela and Siha cells, a significant decrease in proliferation of cells has been noticed after 48h and 72h after TrpRs silencing as compared to the negative control as well as untreated cells but no significant difference was noticed between oh and 24h post transfection (P<0.05). In CaSki cells a significant decrease in cell proliferation was noticed at 24h, 48h and 72h after transfection as compared to that of negative and untreated cells (P<0.05). W/O are untreated and N.C is negative control

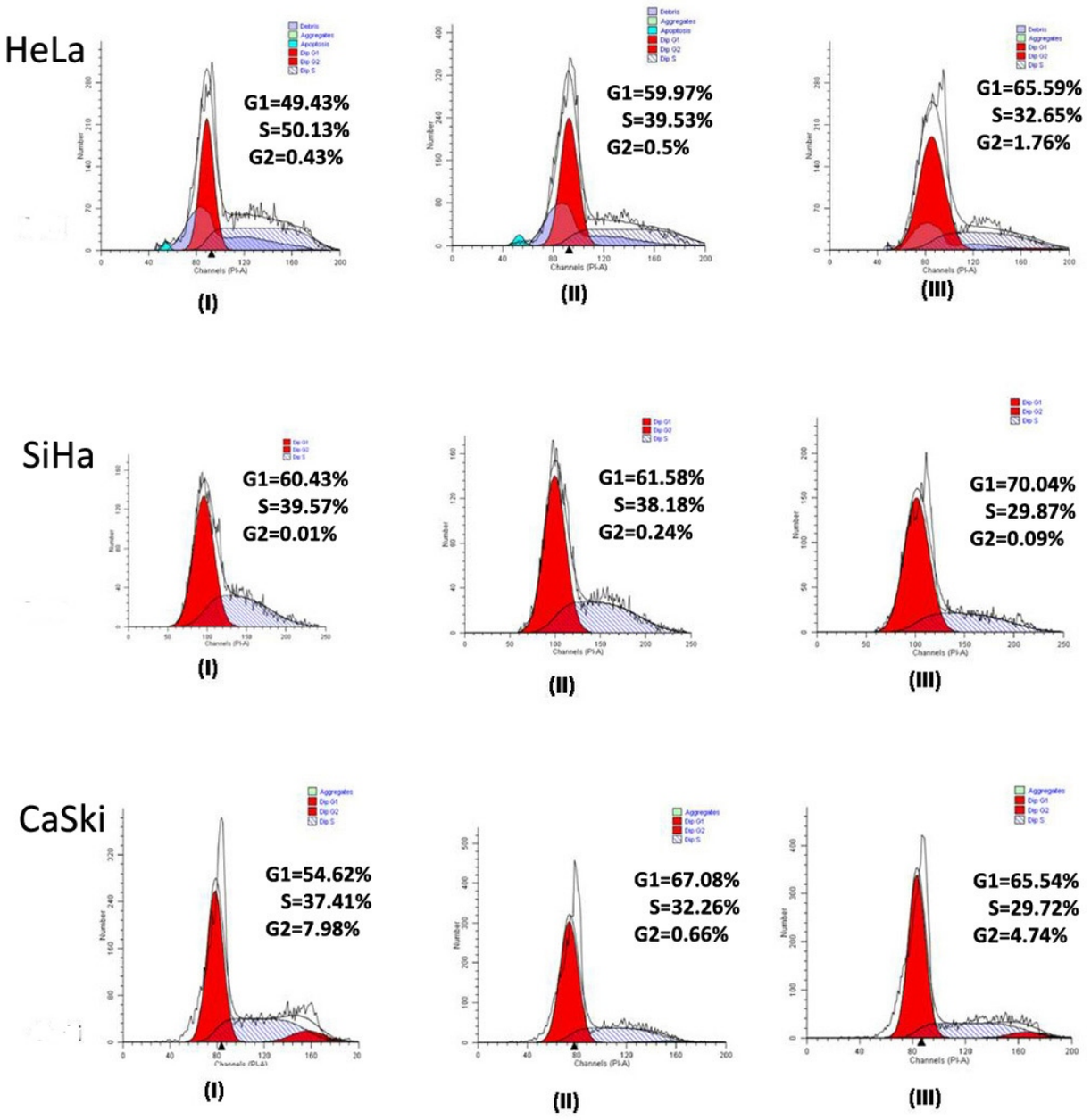

Fig. 5 a: Showing effect of silencing of TrpRS on cell cyclin in HeLa, SiHa and CaSki cells. The number of cells in the S phase was reduced where (I) represents cells without siRNA treatment, (II) represents cells transfected with negative control siRNA and (III) represents cells transfected with siRNA targeting TrpRS

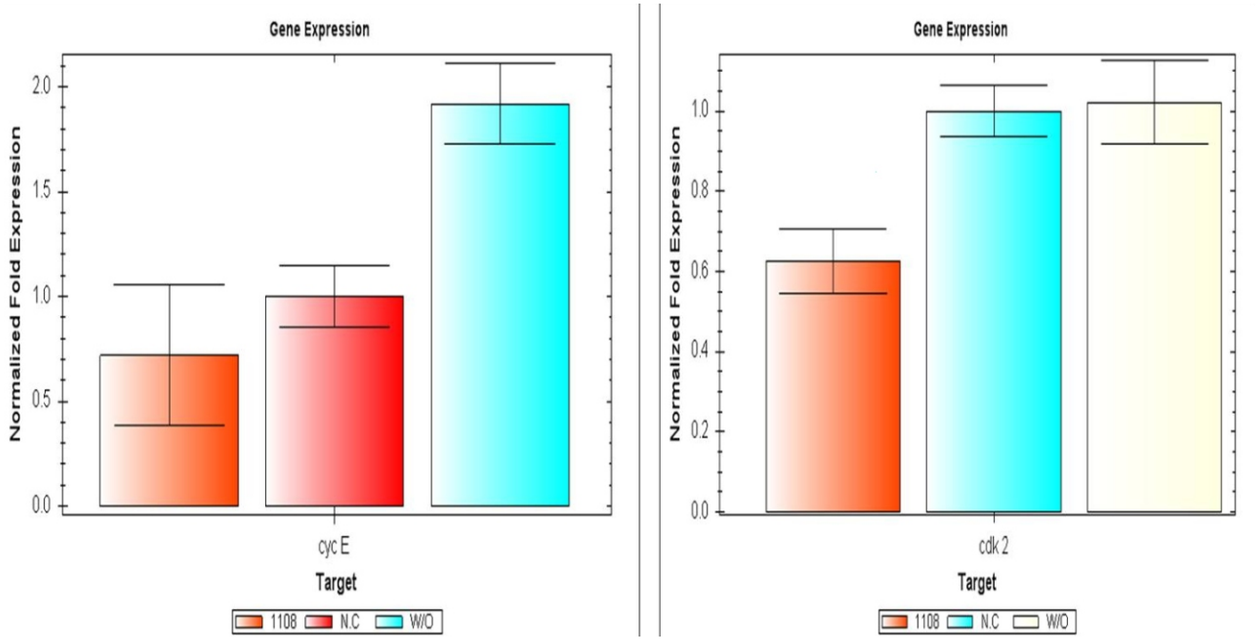

Fig. 5 b: Showing effect of silencing of TrpRS on cyclin E and cdk2 in HeLa cells. Down regulation in the expression of cyclin E and cdk2 has been noticed after silencing as compared to that of negative control as well as untreated cells resulting in reduced number of cells transiting from G1 to S phase W/O are untreated cells and N.C is negative control

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