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CELLULAR & MOLECULAR BIOLOGY LETTERS
http://www.cmbl.org.pl

Received: 16 July 2007
Revised form accepted: 16 November 2007
Published online: 28 December 2007

Volume 13 (2008) pp 240-249
DOI: 10.2478/s11658-007-0050-x

Research article

CAN CYP1A1 siRNA BE AN EFFECTIVE TREATMENT FOR LUNG CANCER?

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Abstract: Previously, we identified a novel correlation between the upregulated expression of telomerase (*hTERT*) and cytochrome P450 1A1 (*CYP1A1*) in A549 human lung cancer cell line. The expression correlation was confirmed by silencing *CYP1A1* expression using siRNA technology and observing a silencing of *hTERT* transcription. Furthermore, silencing *CYP1A1* and subsequently downregulating *hTERT* resulted in the reduction of cancer cell viability by more than 40%, which appeared as early as 24 hours after the treatment. The concomitant downregulation of *CYP1A1* and *hTERT* resulted in rapid cell death. This finding can be further exploited to develop new molecular targets for the treatment of lung cancer.

Key words: siRNA, Gene knockdown, *CYP1A1*, *hTERT*, Transfection

INTRODUCTION

siRNA′s ability for gene silencing has become an increasingly a powerful tool for genetic analysis and a potent therapeutic approach [1, 2]. Moreover, it has been used to study the function of genes associated with human diseases, among which are the down regulation of BCR/ABL oncoprotein in Leukemia [3], inhibition of telomerase by siRNA to increase cell cycle arrest, apoptosis [4] and silencing of IL-12p40 without inducing type 1 interferon to counter inflammatory response [5].

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Abbreviations used: AhR – aryl hydrocarbon receptor; *CYP1A1* – cytochrome P450 1A1; *hTERT* – telomerase

A recent publication from our laboratory has reported novel co-transcription activity between two genes namely; *CYP1A1* and telomerase (*hTERT*) in A549 lung cancer cells [6]. *CYP1A1* is an extrahepatic enzyme which catalyses the bioactivation of polyaromatic hydrocarbons (PAH) constituents abundant in tobacco smoke into mutagens and carcinogens [7, 8]. *CYP1A1* can be induced at the transcriptional level and involves the binding of the inducer to the ligand-activated transcriptional factor aryl hydrocarbon receptor (AhR) [9, 10].
Telomerase plays a critical role in the development of cell immortality and oncogenesis and was frequently reactivated in the lungs of cigarette smokers [11], the expression of its active catalytic component, human telomerase transcriptase (*hTERT*), is present in the majority of lung cancers [12]. A significant correlation has been found between the presence of telomerase activity and current smoking status at the time of diagnosis [13]. Activation of telomerase occurs in the majority of human malignant tumours, however, the relation between telomerase and vulnerability to toxicant activation remains unclear. The presence of telomerase activity in tumours of non-small cell lung cancer patients correlates with a high cell proliferation rate and an advanced pathologic stage [14]. Therefore, it is suggested that telomerase activity is one of the most important prognostic factors in lung cancer patients and that telomerase can be an important target to develop novel therapeutic strategies for the treatment of lung cancers [11]. In this study, we assessed the therapeutic value of silencing *CYP1A1* and subsequent downregulation of *hTERT* on A549 cell proliferation. Furthermore, we investigated the presence of *CYP1A1* and *hTERT* co-transcription in other types of cancer to include glioma and prostate. This approach is aimed at developing siRNA as a future therapeutic tool.

MATERIALS AND METHODS

siRNA designing

Three sets of predesigned *CYP1A1* siRNA duplexes composed of 21-nt were synthesized (Ambion, UK), to include siRNAI 5'-GGCCUGAAGAAUCCACCAGtt-3' (sense) and 5'-CUGGUGGAUUCUUCAGGCCtt-3'; siRNAII 5'-GGAUGAGCCA GCAGUAUGGtt-3' (sense) and 5'-CCAUACUGCUGGCUCAUCCtt-3'; siRNAIII 5'-GGUAUCCAAAAAUGUGUAAtt-3' (sense) and 5'-UUACACAUUUUUGGA UACCtg-3' [15, 16].

siRNA negative control

A double stranded RNA oligonucleotide designed as a negative control was comprised of two 19bp non-targeting sequence with 3'dt overhangs (Ambion, UK). The sequences have no significant similarity to any known gene sequences from rat or human.

Cell culture and transfection

Three sets of human cell lines were used to include; prostate, brain and lung. Normal epithelial prostate cells (CRL-11609) and epithelial carcinoma prostate

cells (CRL-2505) were cultured in modified Eagle's medium essentials (MEME). Glioma and embryonic cell lines used were: astrocytoma (1321N1), glioblastoma astrocytoma (U-87MG), mixed astro-oligodendroglioma (GOS-3), and the control normal embryonic brain (FLOW3000). 1321N1 and GOS-3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, UK), and U-87MG cell line in MEME. The FLOW3000 cell line was cultured in MEM and the normal human astrocytes NHA in astrocyte basal medium (GibcoBRL, UK). Cells were grown in 75 cm^2 Costar plastic flask in monolayer cultures maintained at 37°C in 5% CO_2 humidified atmosphere. The alveolar adenocarcinoma (A549) cells were cultured to subconfluent growth and were trypsinized using trypsin EDTA. A549 cells ($3x10^5$) were subcultured in 4x4 well plates for 24 hrs under ordinary culture conditions before transfection. SiPORT *Amine* (Ambion, UK) mediated transient co-transfection of *CYP1A1* siRNAs was performed in duplicate as previously described [6].

Quantitative RT-PCR (qRT-PCR)

Quantitative RT-PCR was carried out using Roche LightCycler instrument. An average of 1 pg per cell of mRNA was isolated using mRNA isolation kit (Roche, UK). Isolated mRNA (50 ng) was transcribed to cDNA using AMV reverse transcriptase (Roche, UK) and was used as a template for PCR using First strand cDNA Synthesis Kit (Roche, UK). Real time qPCR containing 2 µl cDNA was performed with primers designed using Primer3 software; *CYP1A1* sense 5'CTTGGACCTCCTTGGAGCTG, antisense 5' CGAAGGAAGAGTGTCG GAAG; *hTERT* sense 5'CGTGGTTTCTGTGTGGTGTC; antisense 5'CCTTGTC GCCTGAGGAGTAG; *GAPDH* sense 5' GAGTCAACGGATTTGG TCGT; antisense 5'CGAGATCCCTCCAAAATCAA, in a concentration of 0.5 µM in 20 µl using Fast Strand DNA master ^{PLUS} SYBR Green1 (Roche, UK). After an initial denaturation for 10 min at 95°C, the samples were run for 45 cycles at 95°C (10 s) annealing temperature (*CYP1A1* 56°C, *hTERT* 67°C and *GAPDH* 56°C) (15 s), 72°C (15 s). At the end of each cycle, the fluorescence was measured in a single step in channel F1 (gain 1). All heating and cooling steps were performed with a slope of 20°C/s. The temperature was then raised to 95°C with a slope of 0.1°C/s and fluorescence was measured continuously (channel F1, gain 1) to obtain data for the melting curve analysis [16]. PCR reactions were performed in triplicate and included a negative control (primers without DNA). The expected amplicon size for *CYP1A1*, *hTERT* and *GAPDH* were 212 , 214 and 238bp, respectively.

cDNA copy number calculation

The quantitative amplification was monitored by the level of fluorescence reflecting the cycle number at the detection threshold (crossing point). Absolute quantification is the determination of the absolute amount of target amplicon using an external standard that can be expressed as a copy number or concentration. Genomic DNA can be used as an external standard,

1 µg corresponds to 3.4 x 10^5 copies of single copy gene (QuantiTect-Qiagen user manual). In the present study genomic DNA, of known concentration, was used as a standard DNA and amplified by the LightCycler using a *GAPDH* reference gene in duplicate as recommended (LightCycler user manual). The threshold cycle (Ct) values serve as a tool for calculating the starting template amount of the standard DNA. These were used to plot a standard curve that was used to calculate the copy numbers of unknown samples. To generate a standard curve, five different concentrations of Genomic DNA were prepared in duplicates 0.005 ng, 0.05 ng, 0.5 ng, 5 ng, and 50 ng. A standard curve of average crossing point (Ct) values against concentrations (copy number) was plotted. This graph was used to calculate the amount of unknown copy numbers of the target genes.

Cell viability

Treated plates were subjected to CellTiter-Glo® Luminescent Cell Viability assay (Promega, UK) according to the manufacturer's protocol. Each plate was allowed to equilibrate at room temperature for 30 min before the wells were emptied and 100 µl of fresh media and 100 µl of CellTitre-Glo reagent were added to each well and mixed for 2 min on an orbital shaker to induce cell lysis. The plates were then incubated at room temperature for 10 min before the luminescent signal was detected using Tecan GENios Pro® (Tecan, Austria). Relative luminescence unit (RLU) emitted per cell was plotted against the treatment concentrations.

Statistical analyses

The statistical analysis was performed using SPSS, version 11.0 for Windows. All quantitative data were presented as the mean ± S.D. of three separate experiments and by Student's two-tailed *t* test for group differences and Pairwise comparisons among groups. The $P < 0.001$ was regarded as statistically significant.

RESULTS

The novel constitutive co-transcription of *CYP1A1* and *hTERT* in the alveolar epithelial cancer cell line (A549) was previously reported from our laboratory [6]. In order to assess the role of *CYP1A1* in maintaining the upregulation of *hTERT*, siRNA was used to downregulate *CYP1A1* in A549 cells. Three sets of dsRNA oligo were designed to align various regions of *CYP1A1*. The cells were transfected with the oligos individually using siPROT *Amine* for 24 or 48 hours. QRT-PCR results obtained from these experiments showed that siRNAs I and II delivered to the cells using siPORT *Amine* transfection agent were capable of completely silencing *CYP1A1* transcription after 24 hrs, and siRNA III knocked down *CYP1A1* transcription by 91% ($p<0.001$) [6]. While, siRNA I and III achieved a 100% transcriptional knockdown of *CYP1A1* after 48 hrs transfection, siRNA II downregulated *CYP1A1* by 97% ($p<0.001$). After 24 hr

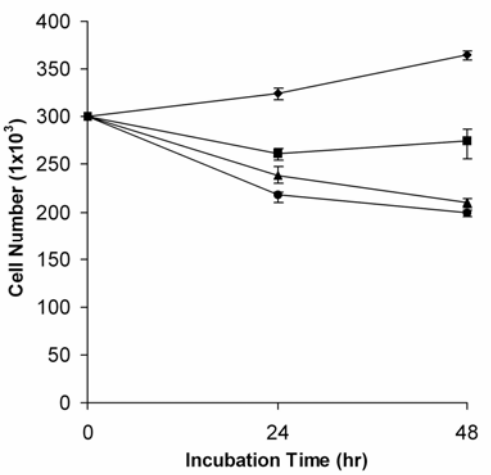

Fig. 1**.** Numbers of cells before and after transfection using siPORT *Amine.* Cell viability was measured before and after CYP1A1 silencing. Three sets of siRNA duplexes used to silencing *CYP1A1*. 4-well plates with 3x10^5 A549 cells/well were transfected with siRNA duplex (I, II, III or control). The control was transfected with the negative oligo (siRNA I: ●, siRNA II: ▲, siRNA III: ■ and Control: ◆). Data represents the mean values ± standard deviation of three individual experiments.

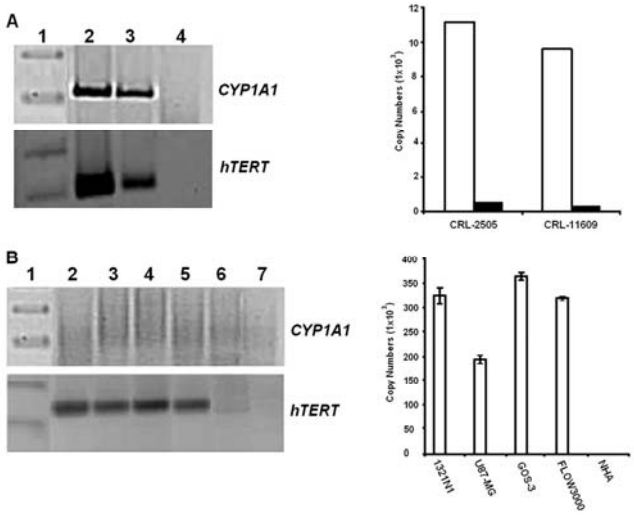

Fig. 2. Constitutive transcription of *CYP1A1, hTERT* in brain and prostate cell lines. *CYP1A1* and *hTERT* transcription profiles were carried out in A – Prostate cell lines; lane 2, represents CRL-11609, and lane 3 is the control normal prostate cells (CRL-2505). Lane 1 is the 100 bp size marker and Lane 4 is the negative control (no DNA). B – Brain cell lines; lanes 2-5 represent 1321N1, U87-MG, GOS-3, FLOW3000, respectively. Lane 6 is the normal brain cell (NHA) as a control. Lane 1 is the 100 bp size marker and Lane 7 is the negative control (no DNA). ■ *CYP1A1,* □ *hTERT.*

CYP1A1 silencing, complete *hTERT* knockdown was observed with siRNA I and III. While silencing *CYP1A1* using siRNA (I and II) delivered with siPORT *Amine*, resulted in *hTERT* transcription being completely diminished after 48 hrs, siRNA III showed low copy numbers of *hTERT* mRNA (4.7%). A negative siRNA control was used to demonstrate that transfection did not induce a non-specific gene transcription.

This study assesses the transcriptional knockdown effect on cell viability. Thus, cell proliferation was determined by cell counting and a reduction in cell number was observed after 24 and 48 hrs of siRNA silencing (Fig. 1).

Prostate and glioma cell lines were used to evaluate the existence of *CYP1A1* and *hTERT* co-transcription in these cell lines and the mRNA of both genes were measured. While both the prostate cancer and normal cell lines transcribed both genes, brain cancer and normal cell lines did not transcribe *CYP1A1,* however, glioma cells and not normal brain cells transcribed *hTERT* (Fig. 2).

DISCUSSION

Previously, we have shown that *CYP1A1* and *hTERT* were transcribed concomitantly in alveolar adenocarcinoma cell line A549 [6]. We confirmed this co-transcription by chemically induced *CYP1A1,* which resulted in the induction of *hTERT*. Furthermore, *hTERT* gene transcription was monitored after siRNA-mediated knockdown of *CYP1A1* transcription in A549, since it is the only cell line that demonstrates co-transcription [6].

In this study, we assessed the efficiency of the *CYP1A1* silencing on *hTERT* activity as a therapeutic approach. Since siRNA is regarded to be highly sequence specific, the most efficient *CYP1A1* downregulation was observed with siRNA I after 24 and 48 hrs, and a similar level of efficacy was achieved with *hTERT*, the co-transcribed gene. The efficiency of siRNA partially depends on the targeted gene mRNA half-life. *CYP1A1* mRNA with its half-life of 2.4 hrs is one of the shortest-lived mRNA studied and is the most unstable of the cytochrome P450 mRNAs. The short half-life appears to be conserved across species, which suggests that this characteristic of the *CYP1A1* mRNA is important for its function [17, 18]. Thus, it would be expected to achieve 100% silencing within 24 hrs. Silencing *CYP1A1* by siRNA I, dramatically attenuated both *CYP1A1* and *hTERT* and confirmed it to be the best silencing target site [1]. The result showed that a 53-57% downregulation of *CYP1A1* by siRNAIII was capable of downregulating *hTERT* by 95.3-97.2%.

To monitor the effect of *CYP1A1* silencing on *hTERT* activity, a cell proliferation assay was performed by counting cell numbers after transfection to determine the effect of *CYP1A1* silencing on cell proliferation. There was a significant reduction in the number of cells after 24hr transfection with *CYP1A1* siRNA ($P<0.001$). siRNA mediated *CYP1A1* silencing and subsequent telomerase transcription downregulation resulted in cell death of more than 40% cancer cells that appeared as early as 24 hrs of transfection with siRNA.

This suggests that *CYP1A1* knockdown affected telomerase activity, which contributes to cell mortality. Telomerase is necessary for the sustained growth of most human tumors and plays an important role in tumourigenesis. Silencing *CYP1A1* decreased *hTERT* transcription and subsequently reduced cell viability. Although Shammas and colleagues showed that the use of a mixture of siRNAs against telomerase led to approximately 77% reduction of telomerase activity within 24 hours and almost complete inhibition after 72 hours, cell death was reported after four weeks [4]. The delay of four weeks that was required for induction of cell death is consistent with the fact that loss of telomerase caused a relatively gradual telomere shortening below the critical value of approximately 2 kbp [19], leading to growth arrest which is associated with replicative senescence and apoptosis [4, 20]. Downregulation of telomerase via the downregulation of *CYP1A1* in lung cancer cells has resulted in rapid cell death. The rapid cell death as a subsequent event(s) of *CYP1A1* and *hTERT* knockdown without a lag phase may be cell specific. Further work was carried out on specific prostate and brain cancer cells, and showed that this co-transcription pattern does not exist in glioma cells. Prostate cancer and control cells both expressed *CYP1A1* and *hTERT* and that co-transcription is unsuitable as a therapeutic approach for glioma and may this be only specific to lung cancer. To further substantiate this finding, the expression of *CYP1A1* and *hTERT* in cells from other organs needs to be tested. In addition, there may be limited application for this strategy to succeed in treating lung cancer due to difficulties in administering and targeting siRNA.

A549 is found to express telomerase and several CYP forms including *CYP1A1* and possesses metabolic activities toward xenobiotics [21-22]. Furthermore, A549 cell line is known to retain several characteristics of human lung epithelial cell *CYP* expression, which makes it a valuable model for mechanistic studies on the induction of the pulmonary CYP system [21-23]. Nonetheless, this work needs to be duplicated utilizing lung tissues.

Further studies exploring the changes in AhR expression, which regulates *CYP1A1* and possibly activate *hTERT* induction, would be required to fully define the signaling pathways leading to apoptosis. AhR is a multiprotein complex containing p23 and chaperon proteins, such as the 90-kDa heat shock proteins (Hsp90). *CYP1A1* expression is controlled through an interaction of the ligand to AhR dissociating Hsp90 and hsp90 co-chaperone p23 molecules [24, 25]. We postulate that a constitutive or induced *CYP1A1* expression associated with elevated AhR activity results in the dissociation of active Hsp90 molecules. Thus, Hsp90 will activate *hTERT* and subsequently will lead to immortalization. The chaperon binding of Hsp90 to *hTERT* is essential in the assembly of the telomerase complex. Although *CYP1A1* and *hTERT* have two different pathways, they may be regulated via the intermediate Hsp90. Previous reports showed that p23 and Hsp90 bind to the *hTERT* subunit of telomerase [26] and a study by Chang and colleagues demonstrated that these two molecules play an important role in the assembly of the active telomerase [27].

The major function of *CYP1A* is to oxidize and enable subsequent conjugation by phase II and elimination. There is evidence suggesting that higher enzyme activity may lead to toxicity and in particular oxidative stress. CYP1A1 has been reported to produce reactive oxygen species during its catalytic cycle. Thus downregulation of *hTERT* via the downregulation *CYP1A1* may prove to be a preventive and a therapeutic target approach rather than the direct downregulation of *hTERT.*

CONCLUSION

The expression of telomerase activity is strongly associated with human cell immortalization and carcinogenesis. Thus, the investigation of the molecular mechanisms that regulate *hTERT* expression may lead to a better understanding of telomerase regulation, cellular senescence, immortalization, and human carcinogenesis. The findings presented in this report correlating *CYP1A1* and *hTERT* transcription, suggests an attractive and possibly an efficient candidate for a new mechanism based on specific gene targets for lung cancer therapy.

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