Research article

CAN CYP1A1 siRNA BE AN EFFECTIVE TREATMENT FOR LUNG CANCER?

KULTHUM MOHAMMED and AMAL SHERVINGTON*
University of Central Lancashire, Department of Biological Sciences, Preston, PR1 2HE, United Kingdom

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].

* Author for correspondence; e-mail: aashervington@uclan.ac.uk, tel.: +44(0)1772 893598
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; \textit{CYP1A1} and telomerase (\textit{hTERT}) in A549 lung cancer cells [6]. \textit{CYP1A1} is an extrahepatic enzyme which catalyses the bioactivation of polyaromatic hydrocarbons (PAH) constituents abundant in tobacco smoke into mutagens and carcinogens [7, 8]. \textit{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 (\textit{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 \textit{CYP1A1} and subsequent downregulation of \textit{hTERT} on A549 cell proliferation. Furthermore, we investigated the presence of \textit{CYP1A1} and \textit{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 \textit{CYP1A1} siRNA duplexes composed of 21-nt were synthesized (Ambion, UK), to include siRNAI 5'-GGCCUGAAGAAUCCACCAGtt-3' (sense) and 5'-CUGGUGGAUUCUUCAGGCCtt-3'; siRNAII 5'-GGAUGAGCCAGUAUGGtt-3' (sense) and 5'-CCAUACUGCUGGCUCAUCCtt-3'; siRNAIII 5'-GGUAUCCAAAAAUGUGUAAtt-3' (sense) and 5'-UUACACAUUUUGGAUACCtg-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 cm² Costar plastic flask in monolayer cultures maintained at 37°C in 5% CO₂ humidified atmosphere. The alveolar adenocarcinoma (A549) cells were cultured to subconfluent growth and were trypsinized using trypsin EDTA. A549 cells (3x10⁵) 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’ CGAAGGAAGAGTTGTCGCAGAAG; hTERT sense 5’CGTGGTTTCTGTGTGGTC; antisense 5’CCTTGTCGCCTGAGGAGTAG; GAPDH sense 5’ GAGTCAACGGATTTGCTCGT; antisense 5’CGAGATCCCTCCTAAAATCAA, 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 siPORT 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 $3 \times 10^5$ 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 tumorigenesis. 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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