Small interfering RNA-mediated suppression of serum response factor, E2-promoter binding factor and survivin in non-small cell lung cancer cell lines by non-viral transfection

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Abstract

OBJECTIVES: Serum response factor (SRF), E2F1 and survivin are well-known factors involved in a multitude of cancer-related regulation processes. However, to date, no suitable means has been found to apply their potential in the therapy of non-small cell lung cancer (NSCLC). This study deals with questions of small interfering ribonucleic acid (siRNA) transfection efficiency by a non-viral transfection of NSCLC cell-lines and the power of siRNA to transiently influence cell division by specific silencing.

METHODS: Different NSCLC cell lines were cultured under standard conditions and transfected, with specific siRNA targeting SRF, E2F1 and survivin in a non-viral manner. Cells treated with non-specific siRNA (SCR-siRNA) served as controls. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed for messenger RNA (mRNA) expression levels. Additionally, transfection efficiency was evaluated by flow cytometry. The analysis of cell proliferation was determined with a CASY cell counter 3 days after transfection with SRF or SCR-siRNA.

RESULTS: Transfection of the NSCLC cell lines with specific siRNAs against SRF, E2F1 and survivin resulted in a very considerable reduction of the intracellular mRNA concentration. CASY confirmation of cell viability demonstrated an excellent survival of the cell lines treated with non-specific siRNA, in contrast to with application of specific siRNA.

CONCLUSIONS: This study reports a reliable transfectability of NSCLC-cell lines by siRNA, initially in a non-viral manner, and a reproducible knockdown of the focussed targets, consequently leading to the death of the tumour cells. This constitutes a strong candidate for a new assessment strategy in the therapy of non-small cell lung cancer.

Keywords: Lung cancer • siRNA • SRF • E2F1 • Survivin

INTRODUCTION

Lung cancer remains a threatening disease, resulting in serious reduction of life quality. Standard therapy consists of a stage-adapted model involving surgery, chemotherapy and radiation, used alone or in combination. Despite some improvement in survival rates after diagnosing lung cancer, it remains a malignant disease with a very poor prognosis. The combination of radio-chemotherapy, followed by surgery, has achieved some success, mainly in advanced stage of the disease.

Standard medication consists mainly of cytotoxic chemotherapy focusing on the interruption of cell division, in which the histological subtype does not play an important role. The standard regimen is almost uniformly structured for all different subtypes. The search for new approaches is therefore of the utmost importance to make further advances in terms of survival following diagnosis, quality of life and progress towards a cure. Very recently, a new group of RNA-based drugs has emerged, which may have tremendous potential in the treatment of malignant tumours [1].

The potential of small interfering ribonucleic acid (siRNA) to interrupt transcription was accidentally discovered in plant experiments. Short RNA sequences that fit complementarily to defined sequences of the targeted mRNA are transfected ex vivo and brought into the cytosol, where they degrade a specific mRNA and thereby silence the targeted gene. It can be seen that, by this highly specific mechanism of action, the application of siRNA is not merely limited to diagnostic and analytical applications, but that it possesses a broader therapeutic potential. Strategies for specific targeting of tumours include the suppression of overexpressed oncogenes, retarding cell division by...
interfering with cyclins and related genes, or enhancing apoptosis by inhibiting anti-apoptotic genes [2].

Past research has demonstrated that siRNA-sequences that are transfected in a cocktail, together with other focusing siRNA-sequences, are as effective in silencing adhesion molecules as single administered sequences [3, 4].

For this investigation, the authors chose two very different targets in varying non-small cell lung cancer lines, which play different roles in the survival of cancer cells. Serum response factor (SRF) is a protein of about 52 kD in weight that is mainly involved in the regulation of early genes and is therefore very little involved in a number of crucial cell regulation systems, as it participates in a variety of cell functions, including cell cycle, apoptosis, cell growth, and cell differentiation [5]. E2F1 has a pivotal role in the cell cycle pathway [6]. Survivin functions as an inhibitor of caspase activation, leading to negative regulation of apoptosis or programmed cell death [7].

The purpose of the present study was to investigate the transfectability of different NSCLC-cell lines, the power of a knockdown of the above-described targets and the resulting impact on cell viability. This approach may have the potential to be studied as a new therapeutic strategy.

MATERIALS AND METHODS

Cell culture

Four non-small cell lung cancer (NSCLC) cell lines were cultured under standard conditions: a) NCI-H460-non-small cell lung carcinoma, b) A549 adenocarcinoma, c) LXF-289 adenocarcinoma and d) SK-MES squamous cell carcinoma (DSMZ–German Collection of Micro-organisms and Cell Cultures, Braunschweig, Germany). Cells were cultured at 37°C and 5% CO₂.

All cell culture media and supplements were purchased from PAA (Colbe, Germany). All cell line media were supplemented with 100 U penicillin and 20 µg streptomycin per ml and with 2 mM L-glutamine. Additionally, the medium was supplemented with 10% FCS (PAA, Colbe, Germany). A549 and SK-MES were cultured in DMEM high-glucose medium, NCI-H460 in RPMI 1640 medium and the LXF-289 cell line in Ham’s F10 medium.

Transfection of siRNAs

One day prior to transfection, 150,000 cells were seeded in 12-well plates for qRT-PCR and FACS and 10,000 cells for CASY analysis for each cell line.

The stated volumes of reagents were calculated for the transfection of one well of a 12-well plate. The appropriate amount of siRNA was diluted in 300 µl of the appropriate basal medium; then 1.1 µl (for 25 nM siRNA concentration) or 1.75 µl (for more than 50 nM siRNA concentration) interferin was added to the mixture. The mixtures were incubated for 20 min at room temperature to form transfection complexes. Next, the growth medium on the cells was replaced by 300 µl of transfection complexes. The cells were transfected for 2 h at 37°C, following which the transfection complexes were replaced by fresh medium, supplemented with FCS, L-glutamine and antibiotics. For the evaluation of the knockdown by qRT-PCR, cells were cultured and transfected in duplicates; for CASY analysis, cells were grown and transfected in quadruplicates.

siRNA sequences

Fluorescein isothiocyanate (FITC)-labelled nonsense-siRNA, for validation of transfection efficiency and SCR-siRNA, was purchased from Qiagen (Hilden, Germany). Qiagen does not provide the sequence of their non-silencing siRNAs but ensures that they have no homology to any known mammalian gene. These non-silencing siRNAs are validated by using Affymetrix Gene Chip arrays and a variety of cell-based assays and have been shown to ensure minimal non-specific effects on gene expression and phenotype.

SRF-siRNA was previously described by Werth et al. [5] with the following sequence: sense 5'-GAUGAGGUUCAGACAAAGGAUGUGCAUGAUCUU-3', antisense 5'-GUUGUGAUGAUCUUCAUCUU-3'; survivin-siRNA (BIRCS): sense 5'-GGACCACCGCAUCUCUACA-3'; antisense 5'-UGAGAGAAGCGGGUGGUCC-3'.

E2F1-siRNA validated by Eurofins: sense 5'-GACGUGUCAGGGACCUUCGU-3'; antisense 5'-ACGAAGGUCUGCAACGUC-3'. All constructs were synthesized by Eurofins MWG Operon, Ebersberg, Germany.

Flow cytometry

Twenty-four hours after transfection with FITC-labelled nonsense-siRNA, cells were washed with PBS (PAA, Colbe, Germany). Afterwards, cells were detached with trypsin and neutralized with TNS (Promocell, Heidelberg, Germany). After centrifuging, the cell pellet was fixed with 2.5% paraformaldehyde in PBS. Flow cytometric analysis (5000 cells/measurement) was performed in a FACScan™ (Becton Dickinson GmbH, Heidelberg, Germany) and evaluated with CellQuestPro software (Becton Dickinson GmbH). The geometric mean values were used for further analyses.

Quantitative real-time PCR

Total RNA from cells was purified 24 h after transfection, using the Aurum™ total RNA mini-kit supplied by Bio-Rad (Hercules, CA, USA). Using Bio-Rad’s iScript™ cDNA Synthesis Kit according to the manufacturer’s instructions, 200 ng of each RNA sample were consecutively reverse-transcribed. Primer design was done using Primer 3 and Primer Premier 5 software (PREMIER Biosoft International, Palo Alto, CA, USA). The following primer sequences were used: GAPDH forward 5’-TCAACAGGCAACCCACTCC-3’; reverse 5’-TGAGTCCACCCCATCGTTG-3’; SRF forward 5’-AGTTCAGCCATCACTG-3’; reverse 5’-AGGATGACGTCTAGTGAGTG-3’; survivin (BIRCS) forward 5’-CTTTTCTTGGAGGCTGC-3’; reverse 5’-TGGGGTCGTCATCTGGC-3’. All primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany). All PCR reactions contained IQ™ SYBR® Green Supermix from Bio-Rad, 400 nM forward and reverse primer and 2 ng of cDNA in a total volume of 15 µl. All PCR reactions were performed in triplicates. Normalized gene expression was calculated by the ΔCt method using GAPDH as a reference.

CASY

For the analysis of cell growth, cell number was analysed three days after transfection using a CASY cell counter system (Scharfe
nonsense-siRNA resulted in a concentration-dependent increasing cell fluorescence, determined by flow cytometry (Fig. 1). The increasing fluorescence was nearly the same in both cell lines.

We therefore decided to transfect all cell lines with 25- and 100 nM siRNA to examine the expression via qRT-PCR. Cells that were analysed for their cell growth were transfected with 50 nM siRNA and analysed by CASY 3 days after transfection.

The transfection of NCI-H460 cells with SRF-, survivin- and E2F1-siRNA leads to a 60%, 30–70% and 60% reduction of the gene expression, respectively (Fig. 2a, b and c). SCR-siRNA transfection did not result in a decreased gene expression in SRF, survivin or E2F1. The silencing of survivin and E2F1 with 50 nM of the corresponding siRNA revealed a reduction in cell growth of more than 50% compared to non-transfected cells (Fig. 2d).

The knockdown of the gene expression in A549 cells after transfection with SRF-, survivin- and E2F1-siRNA was 30%, 70% and 30–80% respectively (Fig. 3a, b and c). However, the transfection of SCR-siRNA achieved no decrease in gene expression, although the expression of survivin and E2F1 increased two- and three-fold after 25 nM SCR-siRNA transfection (Fig. 3b and c).

**RESULTS**

The examination of the transfection efficiency of the NSCLC cell lines NCI-H460 and A549 with fluorescent-labelled nonsense-siRNA resulted in a concentration-dependent increasing cell fluorescence, determined by flow cytometry (Fig. 1). The increasing fluorescence was nearly the same in both cell lines.

We therefore decided to transfect all cell lines with 25- and 100 nM siRNA to examine the expression via qRT-PCR. Cells that were analysed for their cell growth were transfected with 50 nM siRNA and analysed by CASY 3 days after transfection.

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Cell growth was reduced 70% after transfection of survivin- and E2F1-siRNA (Fig. 3d).

The transfection of LXF-289 cells with SRF-, survivin- and E2F1-siRNA resulted in a knockdown of 80–90%; however, the SCR-siRNA transfection also achieved a knockdown of 80% of SRF and E2F2 gene expression (Figs. 4 and 5a, b and c).

A very interesting result is that the transfection of survivin-siRNA in LXF-289 cells resulted in a decrease in cell growth of 90%, whereas E2F1-siRNA transfection decreased the cell growth by only about 50% (Fig. 5d).

SK-MES cells showed equal gene expression after transfection with SRF-, survivin- and E2F1-siRNA, compared to the NCI-H460 cell lines (Fig. 5a, b and c). The transfection of survivin- and SRF-siRNA led to a decrease in cell growth of 80% and 70%, respectively. The transfection of E2F1-siRNA into SK-MES cells shows only a marginal decrease in cell growth.

In all approaches, SCR-siRNA transfection leads to a small decrease of the cell growth, which might be explained by transfection toxicity caused by the transfection reagent, leading to the death of some cells. The transfection of SRF-siRNA revealed no

Figure 3: Relative (a) SRF, (b) survivin and (c) E2F1 expression of A549 cells 24 h after transfection with corresponding siRNAs. The x-axis describes the different concentrations of the siRNAs used; the y-axis reflects the relative expression of the silenced gene, with untransfected cells set to one, while (d) shows % cell number 3 days after transfection with 50 nM siRNAs, compared to untransfected cells, set to 100%.

Figure 4: Relative (a) SRF, (b) survivin and (c) E2F1 expression of LXF-289 cells 24 h after transfection with corresponding siRNAs. The x-axis describes the different concentrations of the siRNAs used; the y-axis reflects the relative expression of the silenced gene, with untransfected cells set to one, while (d) shows % cell number 3 days after transfection with 50 nM siRNAs, compared to untransfected cells, set to 100%.
The current authors are highly interested in the therapeutic application of siRNAs, especially as a medical approach in the therapy of NSCLC. These double-stranded 21–25-nucleotide-long RNAs, which were originally identified as physiological intermediates of the RNA interference pathway, are manufactured by commercial providers and correspond to the mRNA that is to be degraded. In vitro, siRNAs can be directly applied by using cell transfection techniques with cationic lipids. The goal is to reduce cell growth by down-regulating the expression of genes important for cell proliferation, especially in cancer cells. Therefore, the current authors decided to transfect four different cancer cell lines with siRNAs against serum response factor (SRF), E2F1, and survivin.

SRF is a protein that is very little involved in cell proliferation and differentiation; it regulates the activity of many immediate-to-early genes, for example c-Fos, and thereby participates in cell cycle regulation, apoptosis, cell growth, and cell differentiation [8, 9]. E2F1 is a member of the so-called E2F-family, which has, in its role as a transcription factor, important functions in cell cycle and cell reproduction [10]. Survivin has been implicated in both

DISCUSSION

Over recent decades, relatively modest progress has been made in the therapy of NSCLC. Good results have been achieved in an early stage of lung cancer with a small-sized primary lesion, restricted lymph node involvement and where a metastatic disorder can be excluded. Subsequently, surgery can be performed and patients will probably have a good prognosis in terms of survival and relapse-free period.

However, in most cases, diagnosis is made in later stages of lung cancer and, hence, with quite a limited prognosis. Modern therapy regimens include a phase-adapted combination of radiochemotherapy, followed by a surgical intervention or a palliative approach. Despite a quite incriminating therapy with a marked reduction of patients’ life quality, therapy frequently cannot guarantee healing or survival beyond 5 years.

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Table 1: Summary of the siRNAs that mediated potent cell-growth reduction

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>siRNA transfected</th>
<th>Survivin</th>
<th>E2F1</th>
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<tbody>
<tr>
<td>NCI-H460</td>
<td>40–60% reduction</td>
<td>40–60% reduction</td>
<td>60–80% reduction</td>
</tr>
<tr>
<td>A549</td>
<td>Up to 40% reduction</td>
<td>60–80% reduction</td>
<td>60–80% reduction</td>
</tr>
<tr>
<td>LXF-289</td>
<td>Up to 40% reduction</td>
<td>&gt;80% reduction</td>
<td>40–60% reduction</td>
</tr>
<tr>
<td>SK-MES</td>
<td>60–80% reduction</td>
<td>60–80% reduction</td>
<td>Up to 40%</td>
</tr>
</tbody>
</table>

All results are compared to non-transfected cells. Grey boxes indicate siRNAs that reduced cell-growth maximum.
control of cell survival and regulation of mitosis in cancer [11–13]. Survivin is preferentially and highly expressed in cancer cells, with little expression in most normal, non-dividing adult tissues [7, 14, 15]. It is an inhibitor of the apoptotic pathway, thereby inhibiting caspase activity [14]. Survivin expression was found in 53% of tissue sections of 102 patients suffering from non-small cell lung carcinomas [15]. Therefore, survivin turns out to be an interesting target for tumour therapy research.

At first, the present authors were interested in the transfection efficiency of different NSCLC cell lines. To closely emulate clinical application, they decided to work with a cationic lipid as transfection reagent, which already has an optimized protocol procedure [16]. Consequently, good transfectability of the chosen cell lines was found, without a significant cell toxicity, indicating a good tolerance for all other non-tumorigenic cells and tissues.

Silencing of SRF had no high impact on cell growth in the NCL-H460 and the adenocarcinoma cells, but a notable effect on the squamous cell-line.

The silencing of survivin has a recognizable influence on the proliferation of the NSCLC cell lines and was preferentially successful for the A549 and LXF-289 adenocarcinoma cell lines, and the squamous cell carcinoma SK-MES. The present data reflect the results of other research groups, such as Paduano et al. [17].

The present data concerning siRNA-mediated silencing of E2F1 revealed potent antiproliferative effects in the A549 adenocarcinoma cell line and also in the NCI-H-460 non-small cell lung carcinoma. It is highly interesting that the antiproliferative effects of the different siRNAs did not correlate with high knockdown rates in all cell lines tested. Our data show differing responses to the same treatment, which is part of the differential gene expression in malignant cells.

The authors interpret the different responses to the focussed targets within the heterogeneous group of NSCLC cell lines as an outstanding result. Each histological subtype reacts differently to the knockdown of the chosen target. Furthermore, the authors observed different capacities within the same histologic subtype, the adenocarcinomas.

On the one hand, non-viral transfection brings the method near to a clinical application because no security labs are required. On the other, this is a temporary effect, meaning that, for an application in a therapeutic setting, it is highly likely that the siRNA-transfection of the cells would have to be repeated.

The present findings confirm that personalized therapy might be a prerequisite for an effective means of combating lung cancer and that individual gene silencing of specific cancer-related target genes may become a promising new strategy, especially for chemo-resistant tumours. This could be an important precondition for analysing specific qualities of the tumour type, in order to find the perfect therapy for the individual patient.

The overarching aim of the present authors is to establish a new option for NSCLC treatment in the near future, so that these preliminary results can be proven in an in vivo environment.

Conflict of interest: none declared.

REFERENCES

Dr R. Schmid (Berne, Switzerland): I have two questions, one on technical issues. How did you transfect the cell lines? You proposed liposomes for the clinical study in future, but how did you transfect the cell lines in your experiments?

Dr Walker: We used cationic liposomal transfection. The alternative would be a viral vector. We chose this because we wanted to be as near as possible to the clinical setting. With a viral vector you need safety laboratories and therefore we excluded it. With the data I have shown, we achieved a very good transfection rate of the siRNA.

Dr Schmid: The second question, you showed isolated lung perfusion for the tumour, but the tumour isolated to the lung you can resect-

Dr Walker: Perhaps.

Dr Schmid: But what you want to treat is, of course, extended disease. You want to treat the tumour, the disseminated tumour cells, and this is not in the lung you perfused in your model. For the clinical application I think you have to give it systemically.

Dr Walker: Yes, that’s right but perhaps in a lot of clinical settings we see some metastasis in other lobes, therefore it would be a potential application, and let’s take a look at patients who get some chemotherapy before surgery. Perhaps it’s the same setting. I don’t know at the moment. We will have to investigate and wait for what the results will bring.