Experimental Evaluation of Gene Silencing As New Therapeutic Option in the Treatment of Gemcitabine-chemoresistant Non-small-cell Lung Cancer

Mircea Gabriel Stoleriu¹, Andrea Nolte-Karayel¹, Julia Kurz¹, Martin Michaelis², Jindrich Cinatl jr.³, Volker Steger¹, Hans Peter Wendel¹, Christian Schlensak¹, Tobias Walker¹*

¹Department of Thoracic, Cardiac and Vascular Surgery, Tuebingen University Hospital, Germany
²Centre for Molecular Processing and School of Biosciences, University of Kent, Canterbury, UK
³Institute for Medical Virology, Hospital of Goethe University, Frankfurt am Main, Germany

Abstract:
The evolution of technological and therapeutic applications of siRNA since the description of the interference process in 2006 has been extremely rapid and very productive. Currently, at least ten tumor entities and ten viral infections in which siRNA-based therapy might play an auspicious role have been described. Because of the very poor prognosis of NSCLC, we examined and proposed a new therapeutic alternative for the treatment of gemcitabine-resistant lung cancer via siRNA-specific silencing of six important molecules involved in lung carcinogenesis.

Methods: One hundred thousand gemcitabine-chemoresistant A549 cell lines were cultured in a humidified atmosphere containing 5% CO₂ at 37°C and were transfected with specific siRNA targeting HIF1, HIF2, STAT3, SRF, E2F1 and Survivin. The relative expression of these molecules was examined via qRT-PCR and the viability of the chemoresistant cells after siRNA transfection was analyzed using a CASY system 72 hours after specific transfection.

Results: The relative expression of the examined target molecules was suppressed by up to 73% after specific transfection, and the CASY system demonstrated a concentration-dependent reduction in the viability of chemoresistant A549 cells of up to 61%. Therefore the obtained results were significantly better in comparison to the control group.

Conclusions: siRNA complexes may induce accurate suppression of various target molecules involved in lung tumor growth, in particular in gemcitabine-chemoresistant adenocarcinoma. Therefore, siRNA-based nanotechnology might represent a productive platform for the development of new chemotherapeutic agents for advanced stages of lung cancer in the context of a personalized multimodality regimen.

Keywords: Gemcitabine; siRNA; chemoresistance; lung adenocarcinoma
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*Correspondence to: Tobias Walker, Department of Thoracic, Cardiac and Vascular Surgery, Tuebingen University Hospital, Germany. Email: Tobias.Walker@med.uni-tuebingen.de
Introduction

The evolution of siRNA (short intereference RNA) based technology since the description of the interference process published by the two Nobel Prize winners (A. Z. Fire and C.C. Melo) in 2006 has been rapid and highly productive. Within about 7 years, different scientists had described at least ten systemic infections and more than ten tumor entities in which a therapeutic application of siRNA-mediated interference may have a beneficial role. siRNA silencing of various crucial molecules involved in genitourinary, neurological [1], hematological and cardiovascular diseases are currently productive platforms from which to identify new therapeutic options and develop new drugs.

In lung carcinogenesis, current efforts concerning siRNA silencing processes are focusing on early recognition of clinical manifestations of the disease in order to provide an effective curative treatment. Unfortunately, about 40% of patients have metastasis at presentation [1], when most therapeutic options have only a palliative intent [2,3]. In order to develop an effective therapy, a multimodal therapeutic concept including chemotherapy, radiotherapy and surgery [4, 5] is under intensive study. According to the S3 guidelines for lung cancer, chemotherapeutic agents (platinum derivates, taxanes, vinca alkaloids or various monoclonal antibodies) may be used in all stages of the disease [6]. Indeed, the current chemotherapeutic regimen represents the gold standard for the treatment of stage IV, accounting for about 55% of patients [7, 8].

Because chemotherapy is an important component for the treatment of lung cancer, the therapeutic efficiency of the chemotherapeutic agent as well the tumor response to treatment are crucial. An important factor that reduces the efficiency of the treatment in NSCLC (Non small cell lung cancer) is the development of multifactorial chemoresistance. Due to various alterations in drug metabolism and genetic alterations in the DNA [7,9], the chemotherapeutic regimen plays only a modest role in increasing the overall survival rate and quality of life of patients with this disease [10,11]. Therefore, early identification of new targets in the pathogenesis of lung cancer as well as the development of alternative strategies based on the genetic pathways of chemoresistance are pivotal. One of the most studied therapeutic alternatives in various carcinoma cell lines, and in particular in lung cancer, is based on siRNA-mediated posttranscriptional gene silencing.

siRNAs are double-stranded RNA molecules containing 20-25 nucleotides, with an important role in innate immunity and the regulation of gene expression [12,13]. The most important effect of siRNA interference is the transient suppression of various target oncogenes or tumor suppressor genes, thus providing efficient, short-term suppression of gene expression and consecutively of their phenotypic effects [14].

Given the increased chemoresistance of NSCLC cell lines treated with gemcitabine (72%), we proposed an alternative regimen for the treatment of gemcitabine-resistant adenocarcinoma cells via siRNA-mediated specific interference. Experimentally, we induced an individualized siRNA-mediated knockdown of six crucial molecules involved in lung carcinogenesis: HIF 1 (Hipoxia inducible factor 1), HIF2 (Hipoxia inducible factor 2), STAT3, SRF (Serum Response Factor), E2F1 and Survivin. We studied the relative expression of the target molecules after siRNA-mediated interference and measured the viability of the chemoresistant adenocarcinoma cells 3 days after specific siRNA transfection.

Materials and Methods

Cell culture and primary cell isolation

One hundred thousand gemcitabine-chemoresistant A549 cells were cultured in a humidified atmosphere containing 5% CO2 at 37°C in a special high glucose medium (Dulbecco’s Modified Eagle Medium). The cultured media were supplemented with 100 U penicillin, 20 µg streptomycin, 2 mM L-glutamine and 10% conditioned fetal bovine serum (PAA Laboratories, Colbe, Germany).
Transfection protocol of siRNAs

One hundred thousand A549 cells were seeded 24 hours before transfection into 12-well plates for qRT-PCR, along with 20,000 cells for the quantification of surviving cells via CASY analysis (Figure 1). The transfection medium was enriched with different concentrations of interferinTM (1.17 µl at 25 nM siRNA and 1.75 µl at 50 nM and 100 nM siRNA).

The transfection mixture consisted of basal medium, interferinTM (Polyplus, Illkirch, France) and 300 µl of transfection medium containing siRNA. For the CASY experiments, 300 µl transfection medium containing 50 nM specific siRNA was used. All reagents were incubated for 20 minutes at room temperature. Afterwards, the chemoresistant A549 cells were incubated with the siRNA-containing transfection medium for 2 hours at 37°C.

The cells were grown in duplicate for qRT-PCR analysis and quadruplicate for CASY analysis. The studied chemoresistant cells were divided into three different groups:

Group I (control group): Gemcitabine-chemoresistant A549 cells transfected with nonsense siRNA. According to the Qiagen transfection protocol (Hilden, Germany), the transfection reagent enables high transfection efficiency with low cytotoxicity. Furthermore, these nonsilencing siRNAs have been validated using Affymetrix GeneChip arrays and show only minimal nonspecific effects on gene expression.

Group II (scrambled siRNA/siRNA control group): Gemcitabine-chemoresistant A549 cells treated with nonspecific siRNA provided by Qiagen.

Group III (specific siRNA group): Gemcitabine-chemoresistant A549 cells transfected with specific siRNA targeting HIF1, HIF 2, STAT 3, SRF, E2F1 and Survivin.

siRNA sequences

The specific targets were identified using a sense and antisense sequence synthesized and validated by Eurofins MWG Operon, Ebersberg, Germany (Table 1).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Two hundred nanograms of RNA extracted using the AurumTM total RNA mini-kit (Bio-Rad, Hercules, CA, USA) were reverse transcribed using an iSkriptTM cDNA Synthesis Kit 24 hours after transfection. The specific primers (Table 2) were synthesized via software developed by Premier Biosoft International (Eurofins MWG Operon, Germany). In all PCR reactions, the standardized mixture contained a green fluorescent substrate (IQTM SYBR®Green Supermix, Bio Rad, CA, USA), 2 ng of cDNA, and 400 nM forward and reverse primer.

The level of fluorescence was used to quantify gene expression according to the comparative delta Ct method. The average value and the standard deviation were also determined. The GAPDH gene, which is equally expressed in various mammalian cells, was used as a
reference gene to validate the PCR reactions [15].

Table 1 The sense and antisense sequences of the targeted molecules

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Target gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2F1</td>
<td>Sense: 5’GACGUGUCAGGACCUCUUCGU3’</td>
<td>E2F1 gene,</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’ACGAAGGUCUGACACGCUC3’</td>
<td>Chromosome 20 (human)</td>
</tr>
<tr>
<td>Survivin</td>
<td>Sense: 5’GGACCACCGCAUCUCUACA3’</td>
<td>BIRC5 gene,</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’UGUAGAGAUCGGUGUCUUC3’</td>
<td>Chromosome 17 (human)</td>
</tr>
<tr>
<td>STAT3</td>
<td>Sense: 5’GCCUCUCUCUGCAUUUCA3’</td>
<td>STAT3 gene,</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’UUGAAUUCUGCAGAGGC3’</td>
<td>Chromosome 17 (human)</td>
</tr>
<tr>
<td>HIF 1A</td>
<td>Sense: 5’AGAGGUUGGUAAUGUGUGGG3’</td>
<td>BIRC5 gene,</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’CCCCACACAUCCACCUCU3’</td>
<td>Chromosome 14, q21-q24 (human)</td>
</tr>
<tr>
<td>HIF 2A</td>
<td>Sense: 5’AGAUUCCUCGUAUAUGUGU3’</td>
<td>Chromosome 2, p21-p16 (human)</td>
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<td></td>
<td>Antisense: 5’CACCAUAUACGGAACACCA3’</td>
<td>Chromosome 6 (human)</td>
</tr>
<tr>
<td>SRF</td>
<td>Sense: 5’GAUGGAGGUUCAUCGACACAA3’</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’GUUGUCGAUUAACCUCAU3’</td>
<td></td>
</tr>
<tr>
<td>scr siRNA</td>
<td>Not published</td>
<td></td>
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Table 2 The forward and reverse sequences of the targeted molecules

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>E2F1</td>
<td>forward 5’-ACCATCAGTACCTGCGGAGGC-3’</td>
</tr>
<tr>
<td></td>
<td>reverse 5’-ATACCGTGACTTCTCCCCGGG-3’</td>
</tr>
<tr>
<td>Survivin</td>
<td>forward 5’-CTTTCTTGGAGGGCTGC-3’</td>
</tr>
<tr>
<td></td>
<td>reverse 5’-TGCGGTCGTGTCATCTTG-3’</td>
</tr>
<tr>
<td>STAT3</td>
<td>forward 5’-CGGAGAAACAGTTGGGACCCT-3’</td>
</tr>
<tr>
<td></td>
<td>reverse 5’-GAGCTGCTCCAGGATCCGT-3’</td>
</tr>
<tr>
<td>HIF 1</td>
<td>forward 5’-TGCAAGAATGCTCCAGAG-3’</td>
</tr>
<tr>
<td></td>
<td>reverse 5’-GCTGCATGATCTGGTACT-3’</td>
</tr>
<tr>
<td>HIF 2</td>
<td>forward 5’-TGTCGAGACGGAGGC-3’</td>
</tr>
<tr>
<td></td>
<td>reverse 5’-GACGGGCAGTTCCACCTA-3’</td>
</tr>
<tr>
<td>SRF</td>
<td>forward 5’-AGTGCAGGCCATTCACAAGT-3’</td>
</tr>
<tr>
<td></td>
<td>reverse 5’-ACGGATGACGGTCAAGTGGT-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>forward 5’-TGAGGCCAAGACCCTC-3’</td>
</tr>
</tbody>
</table>

**CASY cell confirmation**

The remaining cells were counted 72 hours after transfection using a CASY® Cell Counter (Schä fer System, Reutlingen, Germany). The differentiation and detachment of the dead cells was induced using 500 μl Trypsin/ Ethylenediaminetetraacetic acid and 500 μl Trypsin Neutralization Solution (PromoCell, Germany). The number of nontransfected cells was equivalent to 100% and the number of living cells was used for
further calculation.

Statistical analysis

All data were expressed as mean ± standard error of mean and analyzed using one-way ANOVA with Bonferroni’s multiple comparison test. Special statistical software (GraphPad Prism, La Jolla, USA) was used. Experiments were carried out three times independently with different A549 chemoresistant cell populations and executed in duplicate for qRT-PCR and quadruplicate for CASY measurements.

Figure 2
Relative E2F1 (A), HIF1 (B), HIF 2 (C), SRF (D), STAT3 (E) and Survivin (F) expression of gemcitabine-resistant A549 adenocarcinoma cells 24 hours after transfection with corresponding siRNAs. The X-axis describes the different concentrations of the siRNAs, whereas the Y-axis represents the relative expression of the silenced genes with untransfected cells set to one.

Results

The transfection of A549 cells with E2F1, HIF1, HIF2, SRF, STAT3 and Survivin in gemcitabine-resistant A549 cell lines (Figure 2) led to a moderate reduction in gene
expression (33-37%, 3-9%, 35-73%, 47-69%, 38-53% and 53-62%, respectively).

CASY analysis after a single shot therapy showed a moderate reduction in the viability of gemcitabine A549 chemoresistant cells (cells remaining in SRF group 65%, Survivin group 59%, E2F1 group 48%, STAT3 group 49%, HIF1 group 42% and HIF2 group 39%). The relative expression of the studied molecules was suppressed by up to 73% after specific transfection, and the CASY system demonstrated a concentration-dependent reduction in the chemoresistant A549 cells of up to 61%. Regarding the suppression of the target molecules and the remaining cells after specific transfection, the obtained results were significantly better in comparison to the control group.

Figure 3 Percentage of cells 3 days after transfection with specific siRNA targeting E2F1, HIF 1, HIF 2, SRF, STAT 3 and Survivin, compared with untransfected cells (set to 100%) in A549 cell lines treated with gemcitabine.

Discussion

According to the EUROCARE-4 statistics, the 5-year age-adjusted survival rate for patients with lung cancer diagnosed in 2000-2002 was very poor (10%) [16]. Despite extensive research on new treatment modalities, lung cancer remains the most common cancer worldwide in terms of incidence and mortality in both men and women. Therefore, early diagnosis, effective treatment and adherence to treatment are the most important goals in attempts to prevent the evolution of lung cancer to advanced stages.

As most cases are diagnosed late, research efforts in the area of lung cancer are focusing not only on early detection, but also on new therapeutic strategies for the advanced stages.

According to the S3 guidelines, the therapeutic regimen of lung cancer is based on a multimodal concept including (neo)adjuvant chemotherapy, radiation therapy and surgery in selected patients. Because chemotherapy is a useful component in the treatment of all stages of lung cancer, it is crucial to study the response of the tumor cells to different therapeutic agents, to analyze the multifactorial etiology of chemoresistance, and to suggest new therapeutic
strategies according to mutational analysis of the oncogenes or tumor suppressor genes involved in lung carcinogenesis.

Recent publications demonstrated that the early development of chemoresistance has negative consequences on the effectiveness of treatment and consecutively on survival rate. Furthermore, the prevalence of in vitro chemoresistance of resected NSCLC has increased significantly for first-line chemotherapy agents [10] (cisplatin 63%, carboplatin 68%, doxorubicin 75%) [10]. Chemoresistance has also reached very high levels in patients treated with gemcitabine (72%) [10]. Therefore, we examined a new alternative in the treatment of gemcitabine-chemoresistant adenocarcinoma cell lines involving siRNA-mediated interference.

The same concept was studied by Zhang et al., who discussed the role of miRNAs in lung carcinogenesis, including potential translational clinical implementation [17]. Similar to the experiments performed in other cancer cell lines, we studied a new in vitro model for the treatment of gemcitabine-resistant lung cancer via specific siRNA silencing of six crucial molecules involved in lung carcinogenesis (SRF, E2F1, Survivin, HIF 1, HIF 2 and STAT3 ). SRF is a highly predictive transcription factor that suppresses cell differentiation and growth via the TGF-beta 1 pathway. E2F1 is a highly potent activator of both cell proliferation and p53-dependent / independent apoptosis. Survivin is a potent inhibitor of apoptosis via its regulation of mitosis. The HIF family members are potent transcription factors that promote neoangiogenesis in various carcinoma lines, in particular in lung cancer. STAT3 is a transcription activator that promotes oncogenesis and stimulates tumor suppression depending on the tumor stage in various carcinoma cell lines.

We obtained for each examined molecule individual reliable responses in a concentration-dependent manner, suggesting that the concomitant suppression of these six molecules might be an effective method for preventing the development of chemoresistance through multiple mechanisms of action. This is another argument that supports the concept of individualized multimodal therapy in order to facilitate an effective multi-targeted knockdown of gemcitabine-resistant adenocarcinoma cells.

We proposed a new in vitro model based on A549 adenocarcinoma cells that are similar to adenocarcinoma cells in vivo. These cells grow as a monolayer and ensure the optimal diffusion of nutritional substances. In order to facilitate optimal tumor growth, the A549 cells were cultured on a high glucose medium supplemented with effective nutritional factors. We also used a special transient transfection protocol according to the original publications of Nolte et al. [18, 19] in order to facilitate short-term modification of the relative expression of targeted molecules and to minimize the toxicity to the neighboring healthy cells.

In our in vitro experiments, the carcinoma cells were suppressed after specific siRNA transfection by up to 61%, whereas the gene expression of the target molecules was suppressed by up to 73% in a concentration-dependent manner. This indicates that the selected target molecules might be predictive biomarkers in the treatment of gemcitabine-resistant lung adenocarcinoma. A similar hypothesis was proposed by Ren et al., who demonstrated that the overexpression of RRM1 (ribonucleotide reductase M1) enhances resistance to gemcitabine, suggesting that the mRNA levels of RRM1 could be used as a biomarker in the individual customized therapy of NSCLC [20].

Another study proposed a combination therapy of low-dose gemcitabine and specific PLK1-shRNA (Polo like kinase 1- short harpin RNA) in order to improve the efficiency of the chemotherapeutic agent against the carcinoma cells [21]. Unlike the aforementioned study, we used siRNA-mediated interference to knockdown six other molecules involved in lung carcinogenesis, with reliable and reproducible results. A recent study regarding the chemosensitization of lung adenocarcinoma cells to gemcitabine using a binding protein called 4Ei-1 produced promising results [22]. A similar hypothesis concerning HIF-siRNA mediated chemosensitization was proposed by Minakata et al. [23]. In addition, we demonstrate in our study that HIF is a potent target in gemcitabine-resistant NSCLC. That having been said, we believe that our method offers a platform for further research concerning the chemosensitization of multiresistant adenocarcinoma cells via siRNA-specific multi-targeted interference.
In order to facilitate an optimal knockdown of the target proteins, we conducted the CASY analysis 72 hours after specific transfection to achieve the maximum efficiency possible and to enable a protective short-term process with less adverse effects on the body.

As siRNA interference may only knockdown and not knock out the target molecules [14], we observed a number of surviving gemcitabine-chemoresistant cells (39-65%) after specific transfection, suggesting that this therapy might be successful in terms of a multi-shot transfection strategy in combination with chemotherapy, radiation therapy or surgery.

Our preliminary in vitro results show similarities with those obtained by other groups, suggesting that siRNA-mediated interference might represent a reproducible alternative transient regimen in the treatment of gemcitabine-chemoresistant NSCLC as well an important starting point for further extensive research regarding new therapeutic strategies.

Furthermore, we believe that siRNA-based nanotechnology might provide an accurate individualized tool for the chemosensitization and radiosensitization of (multi-) resistant lung cancer as well for the local transient application of siRNA nanoparticles during various surgical procedures with isolated lung perfusion.

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