Antisense-mediated hTERT Inhibition Specifically Reduces the Growth of Human Bladder Cancer Cells

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ABSTRACT

Purpose: The expression of human telomerase reverse transcriptase (hTERT) is associated with cellular aging and tumorigenesis. It was found in nearly all cancer types but not in most normal, somatic cells. The aim of this study was to investigate whether hTERT inhibition by antisense oligodeoxynucleotides (AS-ODN) can act as an efficient strategy to specifically impair the growth of bladder cancer (BCa) cells in vitro.

Experimental Design: Twenty-three AS-ODNs were designed complementary to five putative single-stranded target sites using a computer-aided secondary structure prediction of hTERT mRNA. The BCa cell lines were transfected once or several times with AS-ODNs, and the influences on cell growth, hTERT mRNA, and hTERT protein levels, as well as on telomerase activity, were examined.

Results: An immediate and continuous reduction of cell viability (up to a complete cell loss) was achieved by treatment with 5 of 23 tested AS-ODNs in EJ28 cells. Additionally, significant inhibition of proliferation (doubling time, clonogenic survival), as well as an induction of G1 arrest, were observed. The specificity of the growth-inhibitory action of the five efficient AS-ODNs was confirmed by diminished hTERT transcript amount (<88%) and reduced hTERT protein content in EJ28 cells. As a consequence, the telomerase activity was inhibited by anti-hTERT treatment ≤60%. Inhibition of viability was shown for an additional three tested BCa cell lines but not for primary fibroblasts after treatment with the five most effective AS-ODNs supporting an antitumor action of these constructs.

CONCLUSION: Specific hTERT inhibition causes remarkable short- and long-term effects on the growth of BCa cells and represents a promising new treatment option of solid tumors. We propose that this alternative treatment could be applied in terms of an instillation therapy.

INTRODUCTION

The induction of hTERT expression results in telomerase activity and contributes, as part of a multistep process, to human carcinogenesis (1, 2). According to this, >90% of examined tumors, including BCa, show telomerase activity (3) in connection with the expression of the activity-limiting component hTERT (4–6). The main function of the ribonucleoenzyme telomerase is the lengthening and capping of the ends of linear chromosomes, the telomeres (7–9). Uncapped or critically shortened telomeres cause cellular responses, such as cell cycle arrest and/or apoptosis (9).

The inhibition of telomerase activity in tumor cells represents a promising anticancer strategy, leading to telomere destabilization and consequently to growth inhibition and/or cell death. The efficacy of this therapy option can be impaired by a lag phase depending on the initial telomere length (10). Many reports describe telomerase inhibition, where effects on tumor cell growth in different solid tumor types were seen after weeks or months of treatment (11–14). Other studies depict immediate cellular responses to telomerase inhibition independent of telomere length (15–18). These immediate effects appear to be caused by deficient telomere capping (9, 17–19).

Both the telomerase RNA component hTR as well as hTERT are essential for telomerase function (20). In contrast to hTR, which is commonly expressed in both normal tissues (without telomerase activity) and cancers, hTERT expression was found solely in tumor cells (5, 21). Therefore, targeting the catalytic subunit hTERT represents a promising approach for diminishing telomerase function that will probably not cause substantial side effects on telomerase negative, somatic cells.

It was shown that transfection with AS-ODNs specifically reduces the expression of target genes (22). This method is based on the complementarity of the constructs to the appropriate target mRNA. Several mechanisms of action exist leading to antisense effects, such as inhibition of transcription, modulation of RNA processing, inhibition of translation, and selective cleavage of the target RNA by the cellular endonuclease RNase H (23). One of the limiting factors, discriminating between efficient and ineffective AS-ODNs, is the accessibility of the

The abbreviations used are: hTERT, human telomerase reverse transcriptase; DT, doubling time; AS-ODN, antisense oligodeoxynucleotide; BCa, bladder cancer; hTR, human telomerase RNA; RT-PCR, reverse transcription-PCR; LF, Lipofectin; NS, nonsense; nt, nucleotide(s); FAM, carboxyfluorescein; ss, single stranded.

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target RNA sequence, because stable secondary structures might prevent AS-ODN hybridization (24). Experimental (“walk the gene,” RNase H mapping), as well as theoretical procedures (secondary structure prediction of the RNA), should increase the probability to find effective AS molecules (25). Several studies about antisense-based telomerase inhibitors targeting hTERT were reported recently (12, 15, 16, 26–28), but only few used the activity-regulating subunit hTERT as a target for empirically selected AS constructs (13, 17, 18, 29, 30).

We used a theoretical, computer-aided secondary structure calculation of the hTERT mRNA (31) to identify putative accessible local target sites for AS-ODNs in a systematic manner. The inhibitory effects of lipid-mediated transfections with anti-hTERT ODNs on growth properties, hTERT transcript, and protein levels, as well as on telomerase activity of the BCa cell line EJ28, were demonstrated in the present study.

MATERIALS AND METHODS

Prediction of Local Single-stranded Motifs within the hTERT mRNA. Because of the size of the hTERT mRNA (4015 nt; accession no. AF015950), the whole sequence was divided into 13 overlapping windows, each 1400 nt in length, and shifted along the RNA at 250 nt steps. For each window, the 10 most probable structures with the lowest free energies (ΔG) were calculated using the mfold software 2.3 (32).

Oligonucleotides, Cell Culture, Transfection Experiments. All ODNs (20-mers except NS-K1 as 18-mer and NS-K5 as 19-mer, each protected by two phosphorothioate bonds at the 3′-ends and 5′-ends, desalted) were synthesized by Invitrogen (Karlsruhe, Germany; Table 1).

The human BCa cell lines EJ28 (33), 5637, J82, and HT1197 (purchased from the ATCC, Manassas, VA) and primary human foreskin fibroblasts were cultivated under standard conditions (37°C, humidified atmosphere containing 5% CO2) in the media according to the provider’s recommendations (Invitrogen). The cells were harvested by trypsin treatment (0.05% trypsin/0.02% EDTA, 5 min, 37°C).

The cells were seeded in appropriate culture plates and grown for 2 days. On the 3rd day, cells were transfected once, or every 2-3 days, with a mixture consisting of ODNs (250 nM) and LF (ratio 1:3 w/w), diluted in serum-free OptiMEM according to the manufacturer’s instructions (Invitrogen). After transfection (incubation for 4 h at 37°C), cells were washed with PBS and incubated in fresh culture medium until additional analyses.

Assessment of ODN Uptake. The transfections of BCa cells with the FAM-labeled NS-ODN NS-K5 were performed as described above in chamber slides (for microscopy) and in six-well plates (for fluorescence activated cell sorter). The proportion of internalized ODN was observed by confocal laser scanning microscopy (Fluorovert FU; Leica, Heidelberg, Germany). Flow cytometry (Becton Dickinson, Heidelberg, Germany) was used to quantify FAM-positive cells at defined times after transfection.

Assays for the Characterization of Antisense Effects. All data were normalized to control cells treated with NS-K1-ODN (34).

Cellular viability was examined 24 and 48 h after the end of transfection using the cell proliferation reagent WST-1 (Roche, Mannheim, Germany).

Doubling times were calculated after harvesting and counting EJ28 cells 24 h after transfection as DT = -ln(nX/n0)/log2 [nX/n0] with nX and n0 being the cell numbers at times X and 0.

 Colony formation assays for EJ28 cells were performed as described previously (35). Briefly, 24 h after the end of the second transfection, 300 cells were plated in triplicates in 25 cm2 flasks and incubated for 7 days. After Giemsa staining, the clonogenic survival was determined by counting all macroscopically visible colonies.

Cell cycle analysis of 2 × 104 two-fold ODN-treated EJ28 cells was performed 24 h after transfection with the CycleTest Plus DNA Reagent Kit (Becton Dickinson), based on DNA staining by propidium iodide using flow cytometry.

Apoptosis was assessed by 4′,6-diamidino-2-phenylindole dihydrochloride DNA staining (1:1000 dilution; Molecular Probes, Eugene, OR). Characteristic nuclear changes like apoptotic bodies were observed by fluorescence microscopy.

Cellular RNA was isolated by a standard protocol at defined times after transfection (Invisorb Spin Cell RNA Mini Kit;Invitek, Berlin, Germany). The hTERT mRNA was quantified by real time RT-PCR using the LightCycler TeloTAGGG

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Nomenclature and sequences of tested AS-ODN complementary to putative hTERT mRNA ss-motifs (accession no. AF015950)</th>
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<tr>
<td>Ma</td>
<td>AS-ODNb</td>
</tr>
<tr>
<td>1037–1056</td>
<td>AS1024</td>
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<tr>
<td>1038–1056</td>
<td>AS1026</td>
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<tr>
<td>1047–1056</td>
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</tr>
<tr>
<td>2328–2336</td>
<td>NS-K5-FAMc</td>
</tr>
</tbody>
</table>

| a Predicted ss-motif (M) within hTERT mRNA. |
| b The name contains the number of the first nucleotide in hTERT mRNA complementary to the AS-ODN. |
| cPhosphorothioates are underlined, and nucleotides complementary to a ss-motif are in bold. |
| d AS-ODN complementary to the hTERT start codon (13). |
| e (34). |
| f /5′-FAM-labeled NS-ODN (15). |
hTERT Quantification Kit and LightCycler instrument (both from Roche).

For the detection of hTERT protein 24 h after transfection, 2 × 10^4 cells were lysed in loading buffer [20% glycerol, 2% SDS, 125 mM Tris (pH 6.8), 5% β-mercaptoethanol, and bromophenolblue], incubated at 95°C for 5 min, and separated on a 7.5% SDS-polyacrylamide gel. Western blot analysis was performed according to a standard protocol using a monoclonal anti-hTERT antibody (NCL-hTERT, 1:250; Novocastra Laboratories, Newcastle, United Kingdom) followed by incubation with a secondary antimouse-horse-radish peroxidase antibody (1:1000; DAKO, Glostrup, Denmark) and an Enhanced Chemiluminescence Kit (Amersham Pharmacia Biotech, Freiburg, Germany).

Telomerase activity in EJ28 cells was assessed by means of the Telomerase PCR ELISA Kit (Roche) using 2.5 × 10^3 cells/reaction following the provider’s instructions.

RESULTS

Secondary Structure Prediction and ODN Design. The characterization of local ss-motifs within the 10 most probable structures of a single sequence window (conservation in percentage) as well as in each of the overlapping windows yielded five ss-motifs in the hTERT mRNA, each being at least nine nt in length. These putative accessible sites with conservative percentage) as well as in each of the overlapping windows were used as target sequences for AS-ODNs. Against each motif, four to five AS-ODNs were designed (Fig. 1; Table 1). Because of the absence of predicted ss-motifs nearby the hTERT start codon, additionally the AtRT-ODN (13) was examined (overall, 23 AS constructs). Furthermore, we tested the control ODN NS-K1 for additional experiments. The AtRT construct showed a modest reduction of viability, independent of the number of consecutive transfections.

DTs and Colony Formation Ability of EJ28 Cells. Because of the immediate effects of the AS-ODN treatment on cell viability, we observed the influence of a single ODN transfection on the DT of BCa cells. Hereby, we could show a concentration-dependent increase in cellular DT, exemplary for three AS-ODN but (as expected) not for the NS control (Fig. 3).

The inhibition of proliferation of anti-hTERT-treated EJ28 cells was confirmed by colony formation assays. The ability of isolated cells to proliferate and generate colonies was clearly reduced after two transfections (Fig. 4). Particularly, cells transfected with AS2315 and AS2206 showed a remarkable decrease in the relative colony number of ≥50%. Furthermore, colonies arising from AS-ODN-treated cells were smaller and less compact than colonies originating from NS-ODN-treated cells (data not shown).

Altering in Cell Cycle Distribution and Apoptosis in EJ28 Cells. The analysis of cell cycle distribution of AS-ODN-transfected EJ28 cells suggested a shift of the percentage of S phase cells toward an arrest in G1 (Table 2).

AS-ODN-treated cells generated from an appropriate monolayer showed no significant differences in apoptosis, as determined by 4’,6-diamidine-2-phenylindole dihydrochloride staining. However, the number of detached cells floating in the culture supernatant after AS2331-ODN treatment was ≥9-fold higher than after NS treatment. Nearly all of these detached cells showed apoptotic bodies (data not shown).

Reduction of hTERT Transcript and Protein Amounts and of Telomerase Activity in EJ28 Cells. To determine the specificity of the growth-inhibitory effects of the AS treatment, we measured the influence on hTERT transcript amounts compared with NS control. Furthermore, we examined whether reduced hTERT transcript numbers resulted in decreased hTERT protein levels and telomerase activities.

We quantified hTERT transcript numbers by real-time RT-PCR at different times after the end of transfection. The five AS-ODNs tested exerted different degrees of mRNA reduction with a respective minimum of relative transcript numbers after 12 h (Fig. 5A). The AS2206-ODN was able to decrease the hTERT transcript level to <20% already 4 h after transfection. The maximal reduction to 12% was shown 12 h after transfection (Fig. 5A). The measurement 24 h after transfection suggested a new increase in relative hTERT mRNA levels in all AS-ODN-treated approaches.

The hTERT protein amount was diminished by each of the
three examined AS-ODNs (ASt2206, ASt2317, and ASt2331) in comparison with the NS control as shown in Western blot analyses 24 h after transfection (Fig. 5B).

The reduction of hTERT transcript numbers, caused by five selected AS-ODNs (ASt2206, ASt2315, ASt2317, ASt2331, and ASt2333), led consequently to an inhibition of telomerase activity. Three AS-ODNs reduced the relative telomerase activity to <50% (Fig. 5C).

**Comparative Evaluation of the Specific Inhibitory Action of hTERT AS-ODNs in Additional BCa Cell Lines and in Nonmalignant Cells.** In three additional BCa-derived cell lines (5637, J82, and HT1197) with detectable hTERT transcript

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**Fig. 2** Effects of different AS-ODN on cell viability of EJ28 BCa cells in dependence of the number of consecutive transfections. The relative viabilities (means of triplicate experiments ±SE or duplicates without error bars) normalized to NS-ODN-treated control cells are displayed.

**Fig. 3** Concentration-dependent extension of DTs. The EJ28 cells were harvested 24 h after transfection and counted, and DTs were calculated (normalized to NS control).

**Fig. 4** Colony formation ability of 2-fold AS-ODN-treated (250 nM) EJ28 cells. After 7 days, the colonies were stained and counted. Data were normalized to NS control and represent means ±SE of triplicates.
The increase in the percentage of G0–G1 cells was determined relative to NS control and shown in brackets.

Table 2:

<table>
<thead>
<tr>
<th>NS control</th>
<th>AS2206</th>
<th>AS2315</th>
<th>AS2317</th>
<th>AS2331</th>
<th>AS2333</th>
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<tr>
<td>G0–G1 (%)</td>
<td>+4.1</td>
<td>53.6</td>
<td>59.1</td>
<td>53.1</td>
<td>49.1</td>
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<tr>
<td>S (%)</td>
<td>55.7</td>
<td>43.3</td>
<td>38.7</td>
<td>43.6</td>
<td>47.5</td>
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<tr>
<td>G2-M (%)</td>
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<td>3.1</td>
<td>2.2</td>
<td>3.3</td>
<td>3.4</td>
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Table 3:

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<th>EJ28</th>
<th>5637</th>
<th>J82</th>
<th>HT1197</th>
</tr>
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<tbody>
<tr>
<td>AS2206</td>
<td>0.48±0.04</td>
<td>0.19±0.01</td>
<td>0.34±0.02</td>
<td>0.88±0.11</td>
</tr>
<tr>
<td>AS2315</td>
<td>0.74±0.02</td>
<td>0.34±0.03</td>
<td>0.87±0.06</td>
<td>0.86±0.05</td>
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<tr>
<td>AS2317</td>
<td>0.43±0.01</td>
<td>0.36±0.04</td>
<td>0.95±0.05</td>
<td>0.84±0.16</td>
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<tr>
<td>AS2331</td>
<td>0.09±0.00</td>
<td>0.15±0.01</td>
<td>0.74±0.06</td>
<td>0.75±0.15</td>
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<tr>
<td>AS2333</td>
<td>0.49±0.02</td>
<td>0.64±0.05</td>
<td>0.81±0.08</td>
<td>0.77±0.09</td>
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DISCUSSION

In our study, we systematically tested, for the first time, a broad spectrum of AS-ODNs–targeted at computationally selected hTERT mRNA ss-sequences–for their ability to specifically inhibit telomerase and growth of human BCa cells in vitro. The results presented herein demonstrate efficient antisense-mediated inhibitory action of 5 of 23 AS-ODNs on the molecular level (hTERT mRNA, hTERT protein, and telomerase activity), as well as on cell growth (viability, DTs, clonogenic survival, induction of a G1 arrest).

The characterization of local target sites by computer-aided secondary structure calculation (31) resulted in the identification of five putative ss-motifs within the hTERT mRNA, e.g., the two sequences 2201–2214 and 2328–2336 (Fig. 1). The theoretical mRNA secondary structure prediction did not include external influences, such as interactions between mRNA and proteins. Nevertheless, all four AS-ODNs complementary to the 2328–2336 motif as well as one construct against the 2201–2214 motif reduced the viability of EJ28 cells effectively already after a single transfection. The localization of the two ss-motifs, one opposite to the other (Fig. 1), appears to cause a destabilization of the hTERT mRNA structure. The subsequent AS-ODN hybridization could allow the dissociation of the double-stranded RNA regions nearby the ss-motifs and explain the efficient inhibitory action of AS constructs targeted at these sites. The long-term treatment led to an amplification of the effects as shown in a consecutively decreasing viability after multiple treatments, culminating in complete cell death caused by four AS2331 transfections (Fig. 2). A possible enhancement of effects by AS-ODN combinations, targeting different sequences within the hTERT mRNA, will be examined in additional experiments.
However, AS-specific and non-AS-specific effects of ODNs can influence cell growth. In contrast to other approaches of AS-mediated telomerase inhibition (12, 13), here we demonstrate an hTERT repression on the mRNA and protein levels, indicating specific interactions between the appropriate AS-ODNs and their target. In addition, telomerase activity—mainly regulated by hTERT—was also diminished.

Because of the short half-life of hTERT mRNA of 50–120 min (37, 38), a rapid turnover could be expected. The respective minimum in relative hTERT transcript numbers in EJ28 cells was detected 12 h after a single transfection (Fig. 5A). Twelve h later, we observed a new increase in hTERT transcript amounts up to levels above that of the NS controls. We hypothesize that the efficient AS-mediated hTERT inhibition could have led to a transcriptional hTERT up-regulation in AS-ODN-treated cells (but not in NS controls). However, the hTERT protein amount was remarkably diminished after 24 h. This could be explained by relatively low numbers of spliced hTERT RNA molecules (0.2–6 transcripts/cell), determined in human fibrosarcoma cell lines (38). A reduction of hTERT transcript number, hTERT protein amount, and telomerase activity after anti-hTERT AS-ODN treatment was also observed by Zhang and He (30) in leukemic cell lines.

An impact on cell growth, caused by specific hTERT targeting, can be induced by two pathways: (a) telomere shortening as a result of repressed telomerase activity (11, 39); and (b) the loss of hTERT-mediated telomere capping (9, 17, 18). We propose that the immediate effects on cell growth shown here are based on the latter mechanism, causing the destabilization of telomeres. Rapid responses of hTERT inhibition on growth of ovarian cancer cells were shown by Saretzki et al. (17) applying ribozymes. The loss of telomere integrity and, as a consequence, the accumulation of DNA damages resulted in cell cycle arrest at G1 and apoptosis (17, 27), as shown here, too. The down-regulation of hTERT expression in G1-arrested cells (38) indicates a causative relationship between cell cycle regulators and telomerase, as confirmed by a p53-mediated hTERT repression in a cell line derived from a squamous cell carcinoma (40). The lowest percentage of proliferating EJ28 cells was seen within the AS2315-treated population and resulted in a remarkably decreased clonogenic survival, because the cells arrested in G1 do not proliferate (Table 2; Fig. 4).

Besides the numerous AS studies showing the suitability of the telomerase RNA component as an efficient target (12, 15, 16, 26–28), few reports indicate the tumor-inhibitory action of a combined antisense-chemotherapy in BCa and possibly also in other tumor types.

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