WT1 and *BCR-ABL* specific small interfering RNA have additive effects in the induction of apoptosis in leukemic cells

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Background and Objectives. The Wilms' tumor gene (*WT1*) is aberrantly over-expressed in leukemic cells. Therefore, we wanted to study the effect of small interfering RNA (siRNA) targeting *WT1* in leukemic cells and normal CD34-positive cells with regard to proliferation, induction of apoptosis, and cell differentiation. Furthermore, we wanted to evaluate whether the additional use of *BCR-ABL* siRNA could increase the anti-leukemic effects of *WT1* siRNA in chronic myeloid leukemia (CML) cells.

Design and Methods. We measured *WT1* expression by reverse transcription polymerase chain reaction (RT-PCR) in various cell lines and in leukemic cells from patients, then transfected the cells with *WT1*-specific and *BCR-ABL*-specific siRNA before carrying out microarray analysis. We used the tunnel assay to measure apoptotic cells.

Results. We observed a reduction of *WT1* gene expression, measured by real-time RT-PCR, in all studied cell lines: K-562, Kasumi-1, MV 4-11 and NB-4, as well as in cells from AML and CML patients. The results also demonstrated that *WT1* siRNA significantly induced apoptosis and inhibited proliferation in MV4-11 cells, NB-4 cells, Kasumi-1 cells ($p<0.01$) and in K-562 cells ($p<0.02$) versus controls. In normal CD34-positive cells, the proliferation was only slightly inhibited (by about 20%) and no induction of apoptosis was found. Combined transfection with *WT1* and *BCR-ABL* siRNA together in K-562 cells increased the inhibition of the rate of proliferation and the rate of induced apoptosis compared to transfection with *BCR-ABL* siRNA or *WT1* siRNA alone ($p<0.01$). We found that most genes involved in cell signaling and protein metabolism were regulated by the *WT1* gene in K-562 cells in a microarray analysis.

Interpretations and Conclusions. In conclusion, *WT1* might be a suitable target for new therapeutic strategies using siRNA in leukemic cells.

Keywords: *WT1*, *BCR-ABL*, small interfering RNA.

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Recent studies have shown that the Wilms' tumor gene (*WT1*) is over-expressed in the majority of patients with acute leukemia and chronic myeloid leukemia (CML).[1] However, the complete function of this gene is not yet understood. What is known is that it encodes a zinc-finger DNA-binding protein with a complex pattern of alternative splicing, resulting in products with different binding specificities and probably different target genes.[2] It has also been reported that the WT1 protein is a transcription factor with mostly repressive activity when bound to the early growth response-1 (EGR-1) DNA consensus sequence, which is present in growth factor gene promoters such as the platelet–derived growth factor A chain (PDGF-A) promoter and insulin-like growth factor II (IGF-II) promoter. Other target genes of *WT1* are the genes for macrophage colony stimulating factor (M-CSF) and transforming growth factor β-1 (TGF-β1).[3-5] But unlike the tumor suppressor gene, *p53*, which is expressed ubiquitously, the expression of the *WT1* gene is not restricted to specific organs. It is also regulated in a time-specific manner.[6] The fact that the *WT1* gene is over-expressed in leukemia prompted some investigators to study its expression in more detail. It has not yet been unequivocally demonstrated that the amount of *WT1* expression measured by quantitative polymerase chain reaction (PCR) correlates with the leukemic burden. Consequently, some investigators concluded that *WT1* gene expression can be used for monitoring residual leukemia after chemotherapy or transplantation. In this context some study groups have enthusiastically defined

WT1 as a *panleukemic marker* due to the fact that it is detected ubiquitously in all distinctive leukemic cells. However, the strong correlation of *WT1* to leukemia raises the question: would silencing the *WT1* gene induce any measurable anti-leukemic effects, as recently shown, for silencing the *BCR-ABL* hybrid gene in chronic myeloid leukemia cells?[8] Since the silencing of genes can now be performed effectively by RNA interference (as described first by Elbashir and co-workers), in this study we aimed to investigate the effects of using small interfering RNA (siRNA) directed against the *WT1* gene in leukemic cells. The definition of RNA interference is: the process of sequence-specific degradation of the targeted mRNA using double-stranded RNA. For RNA interference, synthetic RNA (of 19 to 22 nucleotides in length) are used which are able to mediate cleavage of the target RNA.[7]

In the course of our research we evaluated the anti-leukemic effect of *WT1* silencing in leukemic cells and normal CD34$^+$ cells by studying the proliferation rate, the induction of apoptosis, and the expression of *WT1* mRNA by real-time RT-PCR. Another goal was to study the influence of *WT1* siRNA transfection on cell differentiation. We also tried to find out if the anti-leukemic effect induced by *BCR-ABL* siRNA in CML cells, as demonstrated earlier, can be augmented by the additional application of siRNA directed against the *WT1* gene. Finally, we wanted to evaluate whether it is possible, by use of microarray technique, to screen for genes which are up- or down- regulated in leukemic cells after transfection with *WT1* siRNA in order to find a pattern of the affected genes. This might help us to understand the function of the *WT1* gene better.

Design and Methods

Cell culture

We used the following cell lines in our research: K-562, chronic myeloid leukemia in myeloid blast crisis; Kasumi-1, acute myeloid leukemia FAB M2 with t(8;21); MV4-11, acute monocytic leukemia FAB M5 with t (4;11)(q21;q23); and NB-4, an acute promyelocytic leukemia (FAB M3) with t(15;17). K-562, NB-4 and MV4-11 were purchased from DSMZ, Braunschweig, Germany. The Kasumi-1 cell line was a generous gift from Dr. Nanao Kamada (Hiroshima, Japan). The cells were grown in RPMI 1640 medium (Invitrogen, Heidelberg, Germany) supplemented with 10% fetal bovine serum as described. All cells were maintained in a humidified 37°C incubator with 5% CO_2.

Leukemic cells from patients

Leukemic cells from the bone marrow of four patients were used. CD34$^+$cells derived from bone marrow were obtained from healthy volunteers. All patients and volunteers gave prior, informed written consent to have their cell donations used in the study.

Transfection of siRNA

The siRNA was purchased from Qiagen-Xeragon (Hilden, Germany). The siRNA nucleotides corresponded to nucleotide position 516 to 536 of the human *WT1* gene region (GenBank accession no. NM_024425). The sense and anti-sense were CCACUCAUUCAAGCAUGAGdTT and CUCAUGCUUGAAUGAGUGGdTT, respectively. Sequences of siRNA directed against the BCR-ABL transcript were performed as published previously by Scherr and co-workers.[9] *In vitro* transfections with 0.8 µg siRNA were performed in 24-well plates using the TransMessenger transfection reagent (1×10^5 cells/well) (Qiagen, Hilden, Germany) following the manufacturer's protocol. Transfection of siRNA was performed at least five-fold in each experiment. Each experiment was repeated at least twice.[10] We used a non-silencing siRNA from Qiagen as a control.

RNA isolation and real-time RT-PCR

RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Real time RT-PCR was performed as previously described for *BCR-ABL* and *GAPDH* real-time RT-PCR.[11] The following primers and hybridization probes were used for *WT1* amplification: primers (forward) 5'-GCT GTC CCA CTT ACA GAT GCA-3' and (reverse) 5'-TCA AAG CGC CAG CTG GAG TTT-3', hybridization probes 5'TGT CAG CGA AAG TTC TCC CGG TCC-Fl and LC Red640-ACC ACC TGA AGA CCC ACA CCA GGA CTC ATA CAG-PH. The amount of WT1 PCR product was correlated with GAPDH. All results are given as *WT1/GAPDH* ratios.

Microarray analysis (MA)

We used the GeneChip system (Affymetrix, Santa Clara, CA, USA) for MA. We analyzed K-562 cells twenty-four hours after transfection with *WT1* siRNA and compared them to the controls. MA was performed in triplicate. The targets for GeneChip analyses were prepared according to the current expression analysis technical manual. Approximately 10 µg of total RNA were used as starting material in first strand synthesis using a primer containing the T7 RNA polymerase binding site (5'-GCATTAGCGGCCGCGAAATTAATACGACTCACTATAGGGAGA-(dT)24V-3') (MWG Biotech, Munich, Germany). After generation of double-stranded cDNA from the first-strand cDNA, biotinylated cRNA was synthe-

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sized by *in vitro* transcription using the BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, New York, NY, USA). cRNA was purified using RNeasy columns (Qiagen, Hilden, Germany), fragmented and hybridized to HG-U133A microarrays (Affymetrix, Santa Clara, CA, USA). The arrays were washed, stained and scanned in a GeneArray scanner 2500 (Agilent, Palo Alto, CA, USA). Signal intensities and detection cells were determined using the Microarray Suite (MAS 5.0) software (Affymetrix, Santa Clara, CA, USA). Scaling across all probe sets (global scaling option) of a given array to an average intensity of 1000 was performed to compensate for variations in the amount and quality of the cRNA samples and other experimental variables. Differentially regulated genes were identified by comparing the signal intensities of the indicated groups using the Mann-Whitney rank test at a significance level of 0.05 (Affymetrix Data Mining tool 3.0). All probe sets representing genes of interest for this study were functionally annotated by the NetAffx database (Affymetrix), and HGNC (Hugo Gene Nomenclature Committee) approved gene symbols were used.

Tunnel assay

Apoptotic cells were determined using the *in situ* cell Death Detection kit from Roche Diagnostics (Mannheim, Germany) following the manufacturer's instructions. The apoptotic cells (brown staining) were counted under a microscope. The apoptotic index was defined by the percentage of brown (dark) cells among the total number of cells in each sample. Five fields with 100 cells per field were randomly counted for each sample. We counted a minimum of three samples thus making a total of fifteen single analyses.

Cell proliferation assay

Cell proliferation was determined by 5-bromo-2-deoxyuridine (BrdU) incorporation. Twenty-four hours after transfection of siRNA, the 1×10^5 cells were split into 4-well chamber slides and incubated with a culture medium containing BrdU for 4 hours. BrdU staining was performed using the Roche Kit (Mannheim, Germany) following the manufacturer's instructions. Proliferation was defined as the percentage of brown stained cells among the total number of each sample, and was analyzed in the same way as the apoptotic cells.

Determination of differentiation of leukemic cell lines after WT1 siRNA transfection

Cell differentiation was determined by flow cytometry using the differentiation markers CD13, CD14, CD33, CD34, CD45 and glycophorin as previously described.[12]

Magnetic-activated cell separation

$CD34^+$ cells were positively-selected with anti-CD34 antibody conjugated to iron-dextran microbeads using the MiniMACS device (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

Statistics

Differences in data between the groups were tested by the two-tailed unpaired t-test or Mann-Whitney U-test using the SPSS 10 program (SPSS Inc., Chicago, IL, USA).

Results

Efficiency of transfection

Using fluorescence-marked, non-silencing siRNA (Qiagen) we evaluated the transfection rate in K-562 cells. The number of fluorescence-marked cells was evaluated using a fluorescence microscope twenty-four hours after transfection. For this, we counted 100 cells five times each. We found a mean transfection rate of 70% (range: 61-78%).

WT1 gene expression measured by real time RT-PCR

We found a significant reduction of *WT1* expression in all leukemic cell lines, which was quantified in relation to the housekeeping gene *GAPDH* by real-time RT-PCR (ratio of *WT1/GAPDH*). With this method of analysis, we observed a reduction of *WT1* mRNA levels to amounts between 43% and 72% (mean) after siRNA transfection in the leukemic cell lines K-562, Kasumi-1, MV 4-11 and NB-4 compared to controls (controls were set up to 100%) (Figure 1). Furthermore, we found a reduction of the amount of *WT1* mRNA in leukemic cells derived from AML patients and CML patients compared to in controls. Overall, the amount of *WT1* mRNA was reduced to 35.4% (mean) in the AML patient, 83% in one CML patient and 58% in the other CML patient (mean) as shown in Table 1. Again, we set all controls at 100%.

Inhibition of proliferation and induction of apoptosis in leukemic cell lines and leukemic cells of a CML patient

Twenty-four hours after transfection with *WT1* siRNA we observed an induction of apoptosis in all studied cell lines. We found that 12.1±3.0% of the MV4-11 cells and 13.9±3.2% of the NB-4 cells underwent apoptosis whereas only 6.5±1.5% did so in each control group, which was statistically significant ($p<0.01$). A moderate increase of apoptosis was found in K-562 transfected cells with this rate being 18.5±2.2% compared to 13.5±2.6% in the control group ($p<0.02$). Kasumi-1 control cells had the lowest

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rate of spontaneous apoptosis, with a rate of only 3.8±1.1%, which increased after *WT1* siRNA transfection to 9.6±1.2% (p<0.001) as shown in Figure 2A. The induction of apoptosis decreased concurrently with the proliferation rate of all cell lines in the trials. Proliferation was strongly inhibited by *WT1* siRNA in MV4-11 and NB-4 cells. The proliferation rate decreased from 43.5±2.5% to 14.7±1.4% in MV4-11 cells (p<0.001) and from 43.7±3.1% to 26.3±2.9% in NB-4 cells (p<0.001). The *WT1* siRNA-induced reduction of the proliferation rate in K-562 cells was moderate, in accordance with the induced apoptosis rate with a decrease in the proliferation rate from 49.7±4.2% to 36.9±3.5% (p<0.01). In Kasumi-1 cells the proliferation decreased from 43.1±5.4% to 25.7±3.4% (p<0.001) after transfection with *WT1* siRNA. Transfection with *WT1* siRNA also induced an increased rate of apoptosis (p<0.01) and inhibition of proliferation in cells from a CML patient in myeloid blast crisis (p<0.01) compared to controls, as shown in Figure 2B.

WT1 siRNA effects on induced apoptosis and proliferation in CD34⁺ cells

We found that proliferation in CD34-enriched cells was slightly decreased after transfection with *WT1* siRNA. The proliferation rate of such cells decreased from 29.6±2.8% to 25.6±1.8% (p<0.05). No induction of apoptosis was seen after *WT1* siRNA transfection. The apoptosis rate did not change (12.1±1.6% versus 12.4±1.6%) in controls, as shown in Figure 3.

Additive effects of WT1 siRNA and BCR-ABL siRNA in induction of apoptosis and inhibition of proliferation

As expected, we observed that transfection with *WT1* siRNA alone or *BCR-ABL* siRNA alone inhibited the proliferation rate of K-562 cells compared to controls as shown in Figure 4A (p<0.01 for *WT1* siRNA and *BCR-ABL* each versus control). However transfection with both siRNA together resulted in an exaggerated decrease of proliferation from 49.7±4.2% (controls) to 15.3±1.8% compared to the inhibitory effect of each siRNA independently (28.3±2.8% after transfection with *BCR-ABL* siRNA and 36.9±3.5% after transfection with *WT1* siRNA) as shown in Figure 4A (p<0.01 both siRNA versus each siRNA alone). We also noticed a concurrent additive effect in the induction of apoptosis by *WT1* and *BCR-ABL* siRNA together in K-562 cells. The rate of induced apoptosis increased from 13.5±2.6% (controls) about two-fold to 26.4±3.5%, whereas the use of each siRNA alone was again not as effective (*WT1* siRNA alone 18.5±2.2% and *BCR-ABL* siRNA alone 18.7±2.2%) as shown in Figure 4B.

Additive effects of *WT1* siRNA and *BCR-ABL* siRNA on the induction of apoptosis and inhibition

Table 1A. Effect of anti-*WT1* siRNA on *WT1* mRNA measurement in leukemic cells of three different patients with AML or CML.

	WT1 siRNA In %	*WT1 and BCR-ABL siRNA in %*	*Control in %*	*P*
Female patient with AML M4	35.4±25	ND	100±20	p<0.05
Female patient CML myeloid blast crises	58.2±29	50±28	100±19	p<0.05
Male patient with CML in myeloid blast crises	83.4±32	ND	100±20	NS

Table 1B. Effect of BCR-ABL siRNA on the amount of BCR-ABL gene mRNA.

	BCR-ABL siRNA	*WT1 and BCR-ABL siRNA*	*Control*	*P*
Male patient with bcr-abl positive AML	47.3±17	ND	100±15	p<0.05
Male patient with CML in myeloid blast crises	62.9±21	78.2±24	100±18	NS
Monocytes of CML patient in blast crisis	33.3±19	ND	100±18	NS
Female patient with CML in blast crisis	77.3±21	65.1±23	100±17	NS

WT1 gene expression and BCR-ABL gene expression were measured five times and are shown as the mean (with standard deviation) normalized to the expression of the GAPDH house-keeping gene. The control was set at 100%. All experiments were performed at least twice. WT1 expression decreased after transfection with WT1 siRNA as shown in Table 1A. BCR-ABL expression decreased after transfection with BCR-ABL siRNA. Co-transfection with WT1 or BCR-ABL had no further effects on WT1 expression (Table 1A) or on BCR-ABL expression (Table 1B), respectively.

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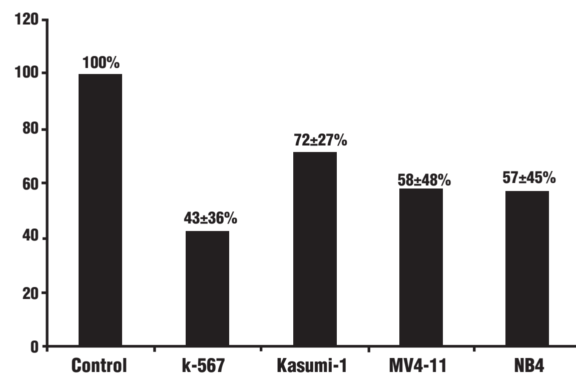

Figure 1. Effect of *WT1* siRNA on the *WT1* gene expression of different leukemic cell lines. *WT1* gene expression was measured by real time RT-PCR and normalized to *GAPDH* expression. Mean and standard deviations are given. The control was set at 100%. Differences between K-562 (43+36%) versus control were significant (p<0.05).

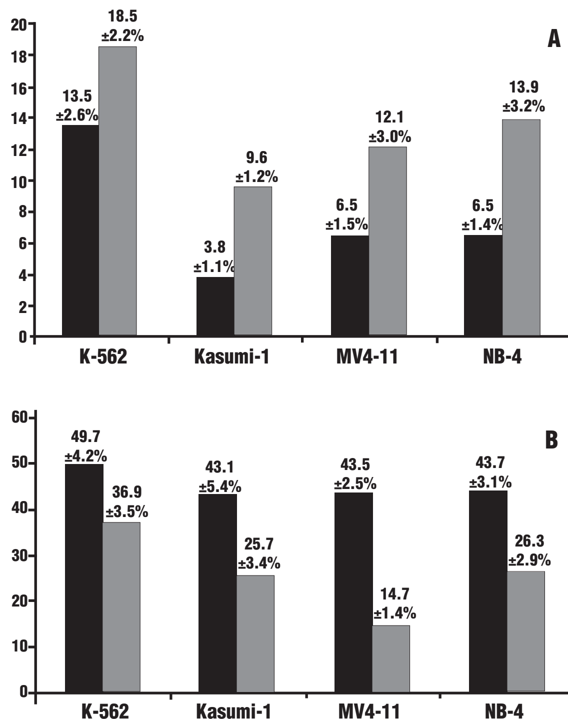

Figure 2. A. Apoptosis rate twenty-four hours after transfection with *WT1* siRNA in K-562, Kasumi-1, MV4-11 and NB-4 cell lines. After transfection with *WT1*-specific siRNA, the induced apoptosis rate of each leukemic cell line increased ($p<0.02$ in the K-562 cell line, $p<0.001$ in Kasumi-1 and $p<0.01$ in others) versus controls. B. Proliferation rate twenty-four hours after transfection with *WT1* siRNA in K-562, Kasumi-1, MV4-11 and NB-4 cell lines. After transfection with *WT1*-specific siRNA, the proliferation rate of each leukemic cell line decreased significantly ($p<0.001$ in all cell lines except K-562 $p<0.01$) versus controls.

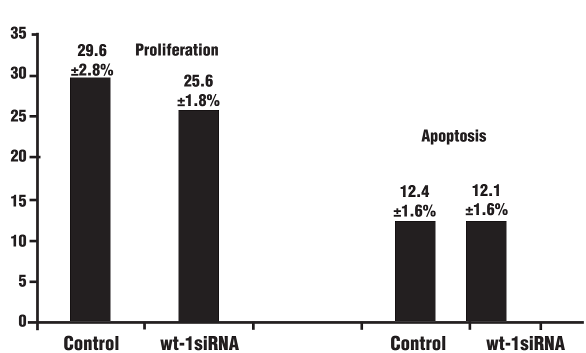

Figure 3. Proliferation rate (left) and apoptosis rate twenty-four hours after transfection with *WT1* siRNA in CD34 positive cells. Controls had a spontaneous proliferation rate of 29.6% (mean) which decreased slightly after transfection with *WT1* siRNA to 25.6% ($p<0.05$). The apoptosis rate (right) after transfection with *WT1* did not change compared to controls.

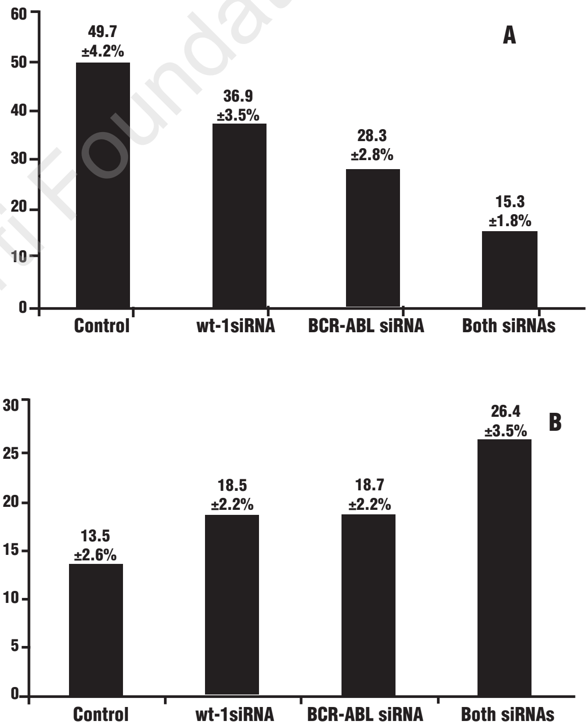

Figure 4. A. Proliferation rate twenty-four hours after transfection with *BCR-ABL* siRNA. *WT1* siRNA and *BCR-ABL* and *WT1* siRNA together in K-562 cells. Controls had a spontaneous proliferation rate of 49.7% (mean) which decreased after transfection with *BCR-ABL* or *WT1* siRNA to 36.9% ($p<0.01$) or 28.3% ($p<0.01$), respectively. Co-transfection of *WT1* and *BCR-ABL* siRNA had additional effects on the inhibition of the proliferation rate as shown in the right column ($p<0.001$ versus control and $p<0.01$ versus *WT1* siRNA or *BCR-ABL* siRNA alone). B. Apoptosis twenty-four hours after transfection with *BCR-ABL* siRNA, *WT1* siRNA and *BCR-ABL* and *WT1* siRNA together in K-562 cells. Controls had a spontaneous apoptosis rate of 13.5% (mean) which increased after transfection with *BCR-ABL* or *WT1* siRNA to about 18.5% ($p<0.02$) or 18.7% ($p<0.02$). Co-transfection of *WT1* and *BCR-ABL* siRNA had additional effects on the apoptosis rate, as shown in the right column ($p<0.01$ both siRNA versus control and $p<0.02$ versus *WT1* siRNA or *BCR-ABL* siRNA alone).

of proliferation were also seen in CML cells of a female patient in myeloid blast crisis as shown in Figures 5A and 5B.

However, co-transfection with *WT1* and *BCR-ABL* siRNA had no additional effects on the *BCR-ABL* mRNA levels or *WT1* mRNA levels as measured by real-time RT-PCR. The BCR-ABL mRNA amount was not altered when *WT1* siRNA was added to *BCR-ABL* siRNA (70% *BCR-ABL* siRNA alone versus 79% *BCR-ABL* and *WT1* siRNA compared to control [set at 100%]) as shown in Table 1B. The effect on *WT1* mRNA amounts is similar, and was unaltered by adding *BCR-ABL* siRNA, as shown in Table 1A.

Microarrays

A number of genes were affected by *WT1* siRNA. In this study, we considered only the 25 most down- or up-regulated genes which show a marked change in expression (fold change < 0.55 for down-regulated and >2.39 for up-regulated). We found that most genes involved in cell signaling (ARHDIA, Rho GDP

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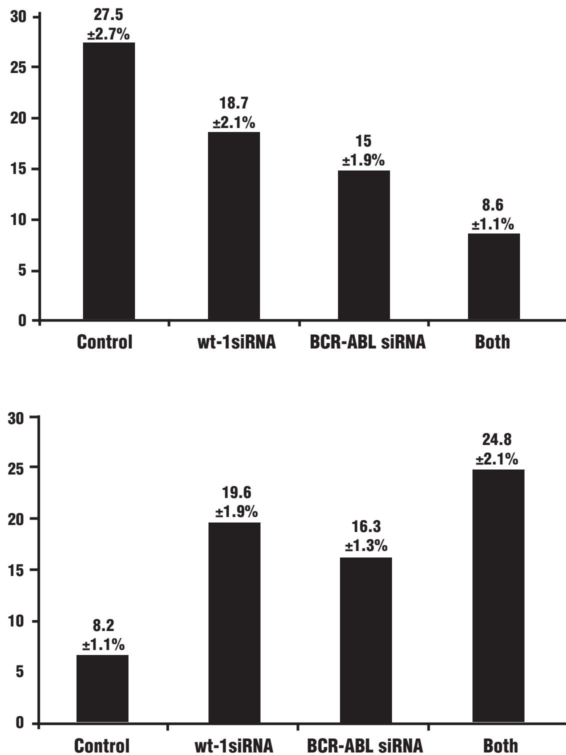

Figure 5. A. Proliferation rate after transfection with *BCR-ABL* siRNA, *WT1* siRNA and *BCR-ABL* and *WT1* siRNA together in a CML patient in blast crisis (UPN 3). Controls had a spontaneous proliferation rate of 27.5% (mean) which decreased after transfection with *BCR-ABL* or *WT1* siRNA to 15% (p<0.01) or 18.7% (p<0.01), respectively. Co-transfection of *WT1* and *BCR-ABL* siRNAs had additional effects on the inhibition of the proliferation rate, as shown in the right column (p<0.001 versus controls and p<0.01 versus *WT1* siRNA or *BCR-ABL* siRNA alone). B. Apoptosis rate after transfection with *BCR-ABL* siRNA, *WT1* siRNA and *BCR-ABL* and *WT1* siRNA together in a CML patient in blast crisis (UPN 3). Controls had a spontaneous apoptosis rate of 8.2% (mean) which increased after transfection with *BCR-ABL* or *WT1* siRNA to 16.3% (p<0.01) or 19.6% (p<0.01), respectively. Co-transfection of WT1 and *BCR-ABL* siRNA had additional effects on the inhibition of the proliferation rate, as shown in the right column (p<0.001 versus controls and p<0.01 versus *WT1* siRNA or *BCR-ABL* siRNA alone).

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dissociation inhibitor α, prostaglandin E receptor 3, angiotensin II receptor), and ribosomal protein synthesis (ribosomal protein S20, mitochondrial ribosomal protein 64) were down-regulated, whereas genes responsible for small protein synthesis and metabolism (NP220 nuclear protein, zinc finger proteins 292, M-phase phosphoprotein, swap-70 protein, protease 24) were mostly up-regulated in K-562 cells. Figure 5B and Tables 2A and 2B show the MA with the most down-regulated and up-regulated genes.

WT1 siRNA and differentiation of CD34⁺ cells and leukemic cell lines

Another goal of this study was to evaluate whether the silencing of the *WT1* gene might induce effects in cell differentiation in the transfected cells. We used CD13, CD14, CD33, CD34, CD45 and glycophorin as differentiation-specific surface markers. We observed no effects on differentiation after transfection with *WT1* siRNA in CD34 positive cells and in leukemic cell lines K-562, Kasumi-1, MV4-11 and NB-4 (*data not shown*).

Discussion

The discovery that siRNA could be delivered effectively into cells of mammals raises the possibility that selective intervention in leukemic cell gene regulation might be feasible for the treatment of leukemia. Our research demonstrates that the use of siRNA directed against the *BCR-ABL* fusion gene inhibits *BCR-ABL* gene expression. Moreover, the first report on *BCR-ABL* siRNA from Wilda and co-workers showed that *BCR-ABL* silencing was accompanied by strong induction of apoptotic cell death.[8] The rate of induced apoptosis was even as high as that induced by 1 μM imatinib. Other studies have confirmed these effects of *BCR-ABL* siRNA in CML cells.[9-10] Furthermore, Wohlbold *et al.* demonstrated that transfection with *BCR-ABL*-specific siRNA increased the sensitivity to imatinib in both the imatinib-sensitive and imatinib-resistant CML cell line.[10]

Our study began by examining the effects of siRNA directed against *WT1* transcripts. *WT1* was chosen as a target for gene silencing because it is aberrantly overexpressed in almost all leukemic cells, regardless of the type of leukemia, whereas *WT1* expression levels in normal hematopoietic progenitor cells are less than one-tenth of the expression levels in leukemic cells.[13,14] Moreover, it has been shown that *WT1* is active during hematopoiesis and regulates the proliferation and differentiation of blood cells,[15] so the silencing of this gene might interfere with the proliferation of leukemic cells. Recently, the WT1 protein has also been under investigation as a promising tumor antigen for immunotherapy against leukemia and various other kinds of tumors, including lung and breast cancer.[16] Preliminary studies show that synthetic peptides derived from WT1 proteins sensitize human T lymphocytes to recognize leukemic cells and thus, might open the door to WT1-based immunotherapies.[16-19]

We found that siRNA directed against the *WT1* gene inhibited *WT1* gene expression in three AML cell lines and one CML cell line. *WT1* expression was significantly reduced in leukemic cells of two AML and two CML patients after transfection with WT1 siRNA versus controls. But we observed differences in the reduction of *WT1* gene expression measured by real-time RT-PCR in each of the tested cell lines. Compared to controls (whose results we set at 100%), we measured

Table 2A. **Microarray analysis with the most down- and up-regulated genes.**

AffyID	*Gene symbol*	*Location*	*Title*	*Fold change*
201167_x_at	ARHGDIA	Chr:17q25.3	Rho GDP dissociation inhibitor v	0.333
213933_at	PTGER3	Chr:1p31.2	rostaglandin E receptor 3 (subtype EP3)	0.43
205390_s_at	ANK1	Chr:8p11.1	ankyrin 1, erythrocytic	0.454
216247_at	RPS20	Chr:8q12	ribosomal protein S20	0.472
205357_s_at	AGTR1	Chr:3q21-q25	angiotensin II receptor, type 1	0.485
206291_at	NTS	Chr:12q21	neurotensin	0.486
206064_s_at	PPIL2	Chr:22q11.21	peptidylprolyl isomerase (cyclophilin)-like 2	0.488
204387_x_at	MRP63	---	mitochondrial ribosomal protein 63	0.499
208930_s_at	ILF3	Chr:19p13.2	interleukin enhancer binding factor 3, 90kDa	0.506
207425_s_at	MSF	Chr:17q25	MLL septin-like fusion	0.513
202984_s_at	BAG5	Chr:14q32.33	BCL2-associated athanogene 5	0.522
209088_s_at	UBN1	Chr:16p13.3	ubinuclein 1	0.523
207643_s_at	TNFRSF1A	Chr:12p13.2	tumor necrosis factor receptor superfamily, member 1A	0.528
215357_s_at	PDIP46	Chr:22q13.31	polymerase delta interacting protein 46	0.528
217857_s_at	RBM8A	Chr:1q12	RNA binding motif protein 8A	0.529
211576_s_at	SLC19A1	Chr:21q22.3	solute carrier family 19 (folate transporter), member 1	0.529
202238_s_at	NNMT	Chr:11q23.1	nicotinamide N-methyltransferase	0.533
202045_s_at	GRLF1	Chr:19q13.3	glucocorticoid receptor DNA binding factor 1	0.533
200659_s_at	PHB	Chr:17q21	prohibitin	0.537
209645_s_at	ALDH1B1	Chr:9p11.1	aldehyde dehydrogenase 1 family, member B1	0.544
32029_at	PDPK1	Chr:16p13.3	3-phosphoinositide dependent protein kinase-1	0.545
201109_s_at	THBS1	Chr:15q15	thrombospondin 1	0.545
215838_at	LIR9	Chr:19q13.4	leukocyte Ig-like receptor 9	0.547
202678_at	GTF2A2	Chr:15q21.3	general transcription factor IIA, 2, 12kDa	0.55
211697_x_at	LOC56902	Chr:2p13.3	putative 28 kDa protein	0.55

Table 2B. **Microarray analysis with the most down- and up-regulated genes.**

AffyID	*Gene symbol*	*Location*	*Title*	*Fold change*
213074_at	IRAK1BP1	Chr:6q14-q15	interleukin-1 receptor-associated kinase 1 binding protein 1	2.391
214130_s_at	PDE4DIP	Chr:1q12	phosphodiesterase 4D interacting protein (myomegalin)	2.392
212815_at	RNAH	Chr:6q16	RNA helicase family	2.415
212381_at	USP24	Chr:1p32.3	ubiquitin specific protease 24	2.424
200843_s_at	EPRS	Chr:1q41-q42	glutamyl-prolyl-tRNA synthetase	2.428
201291_s_at	TOP2A	Chr:17q21-q22	topoisomerase (DNA) II α 170kDa	2.432
210732_s_at	LGALS8	Chr:1q42-q43	lectin, galactoside-binding, soluble, 8 (galectin 8)	2.445
203895_at	PLCB4	Chr:20p12	phospholipase C,β 4	2.45
217952_x_at	PHF3	PHF3	PHD finger protein 3	2.486
214129_at	PDE4DIP	Chr:1q12	phosphodiesterase 4D interacting protein (myomegalin)	2.486
203301_s_at	DMTF1	Chr:7q21	cyclin D binding myb-like transcription factor 1	2.555
215388_s_at	HFL1	Chr:1q32	H factor (complement)-like 1	2.565
212945_s_at	MGA	Chr:15q15	MAX gene associated	2.578
209306_s_at	SWAP70	Chr:11p15	SWAP-70 protein	2.603
215731_s_at	MPHOSPH9	Chr:12q24.31	M-phase phosphoprotein 9	2.606
214709_s_at	KTN1	Chr:14q22.1	kinectin 1 (kinesin receptor)	2.623
214716_at	BMP2K	Chr:4q21.23	BMP2 inducible kinase	2.674
216449_x_at	TRA1	Chr:12q24.2-q24.3	tumor rejection antigen (gp96) 1	2.761
212593_s_at	PDCD4	Chr:10q24	programmed cell death 4 (neoplastic transformation inhibitor)	2.767
214766_s_at	ELYS	Chr:1q44	ELYS transcription factor-like protein	2.861
212388_at	USP24	Chr:1p32.3	ubiquitin specific protease 24	2.982
204297_at	PIK3C3	Chr:18q12.3	phosphoinositide-3-kinase, class 3	2.986
212368_at	ZNF292	Chr:6q15	zinc finger protein 292	3.136
213775_x_at	NP220	Chr:2p13.2-p13.1	NP220 nuclear protein	3.283
209902_at	ATR	Chr:3q22-q24	ataxia telangiectasia and Rad3 related	3.812

All genes were significant with $p<0.05$ fold difference (FC) in mean gene expression levels for transfected K-562 cells with WT1 siRNA and controls. Only FC values of <0.55 for down-regulated genes and FC values >2.39 for up-regulated genes are considered in the Table.

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a marked reduction in *WT1* gene expression to 43% in K-562 cells and a lower grade of reduction to 72% in Kasumi-1 cells. These amounts might also reflect the variation of effectiveness of transfection with *WT1* siRNA in these cell lines, which we found to vary between 38% and 75%.

It is reported that *WT1* can interfere with induced differentiation in leukemic cell lines, suggesting that its expression is associated with the maintenance of a primitive phenotype and giving it a role in leukemogenesis.[18] However, we observed that cell cycle phase distribution was not affected by *WT1* expression. Furthermore, no signs of impaired differentiation (as measured by the expression of surface markers CD11b, CD14, and glycophorin) were described earlier by Svedberg and co-workers.[18] In accordance with these findings, we also did not find any changes in the expression of the surface markers CD13, CD14, CD33, CD34, CD45 and glycophorin twenty-four hours after transfection with *WT1* siRNA in all studied cell lines, nor in normal CD34+ progenitor cells. One remarkable result was that the proliferation rate of all examined cell lines declined significantly after transfection with *WT1* siRNA. The inhibition of proliferation was most effective in the monocytic leukemia cell line MV4-11 and least effective in the K-562 cell line. Transfection of *WT1* siRNA into cells of a CML patient in blast crisis also induced a significant decrease in the proliferation rate. Besides the inhibition of proliferation, apoptosis was induced in all cell lines, and varied from about 40% in K-562 cells up to 250% in the Kasumi-1 cell line. However, the degree of induced apoptosis did not strictly correlate with the grade of inhibition in proliferation in the examined cell lines. We speculate that this might indicate that the process of apoptosis is not uniform in the leukemic cell lines which we examined here, but influenced by more complex mechanisms. Since each leukemic cell type varies in its chromosomal aberration with different specific leukemic-specific oncoproteins and shows different patterns of response to chemotherapy, it might very well be possible that the induction of apoptosis though the *WT1* gene is also influenced differently.

As shown here for siRNA, antisense oligomers against *WT1* were able to reduce WT1 protein expression in leukemic cells and inhibit growth of leukemic cells, as reported earlier.[19] Although siRNA and antisense oligomers are directed against the same target mRNA, it must be noted that they have different pathways to cleave mRNA. The binding of siRNA to the RNA-induced silencing complex (RISC) activates and cleaves the target mRNA, whereas antisense oligomers block translation of the mRNA or induce its degradation by the enzyme RNase H. siRNA are thought to be much more effective than antisense oligomers in the degradation of their target mRNA.[20]

Interestingly, we found that *WT1*-specific siRNA did not induce apoptosis in normal CD34+ progenitor cells, but caused only a moderate decrease of about 20% in the proliferation rate. This may be due to a much lower level of *WT1* gene expression in normal CD34 cells.[14,21,22] The fact that *WT1* siRNA induced apoptosis mainly in leukemic cells qualifies *WT1* siRNA as a therapeutic agent in acute leukemia. It also demonstrates that the *WT1* gene could be considered more as an oncogene than a tumor suppressor gene in leukemic cells, as discussed recently in other research.[17]

In the microarray analysis we found that most genes involved in cell signaling and ribosomal protein synthesis were down-regulated, while genes responsible for small protein synthesis and metabolism were mostly up-regulated in K-562 cells. Further studies with real-time PCR are necessary to confirm the results of our microarray analysis.

It is also notable that we found additive effects in the rate of induced apoptosis and the rate of inhibition of proliferation by transfection with *WT1* and *BCR-ABL* siRNA together in *BCR-ABL* positive K-562 cells and *BCR-ABL* positive cells from a CML patient. The rate of induced apoptosis increased additionally about 50% in these cells versus those transfected with *WT1* siRNA or *BCR-ABL* siRNA alone (p<0.001). Concurrently, the proliferation rate also decreased by about 50% compared to that when only a single siRNA was used (p<0.001). Therefore, both siRNA affected CML cells by different pathways, targeting different oncogenes. Leukemic cells with disease-specific fusion genes, such as the *BCR-ABL*-positive CML cells, may be optimal targets for this purpose. It may be possible to increase the anti-leukemic effect of a single siRNA directed against leukemic specific fusion genes, e.g. *AML1-ETO*, *PML-RARA* or *CBFB-MYH11*, by transfection with additional *WT1* siRNA. The use of several siRNA in order to induce additive effects towards target cells has already been described in other systems.[23-25] Unlike other researchers, we did not use electroporation to deliver siRNA into the cells. We used liposomal reagents instead (although it is easier to deliver siRNA into cells in suspension by electroporation rather than using transfectional reagents). The induced apoptosis rate and inhibition of proliferation was about 10-20% lower in our study than in studies using electroporation for *BCR-ABL* siRNA experiments.[8-10] Delivering siRNA safely into human cells remains an important unsolved question for future treatment of leukemia patients with siRNA.

Our findings show that not only can *WT1* siRNA induce apoptosis and inhibition of proliferation of leukemic cells, but also that it has additive effects with *BCR-ABL*-specific siRNA. The transfer of sever-

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al siRNA, including *WT1*-specific siRNA, could offer new therapeutic strategies for the treatment of leukemia.

AHE, MK, RP, LK-H, HO, DWB, BO: substantial contributions to: conception and design, or acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; and 3) final approval of the version to be published.

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