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# Wilms' tumour gene 1 (WT1) as a target in curcumin treatment of pancreatic cancer cells

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ABSTRACT

The transcription factor WT1 plays an important role in cellular proliferation and survival of various cancer cells, and is frequently expressed in pancreatic cancer. Curcumin has been shown to be a potentially effective agent in pancreatic cancer. In this context, the purpose of this study was to determine the role of WT1 in a curcumin-treated pancreatic cancer cell line.

To study the effect of curcumin on the expression of WT1, we incubated the pancreatic cancer cell line PANC-1 with different amounts of curcumin. The expression of WT1 on mRNA and protein level was measured with real-time RT-PCR and Western blot analysis. The incubation of the pancreatic cancer cell line PANC-1 with curcumin resulted in an inhibition of cellular proliferation as measured with MTT assay. The expression of WT1 on mRNA and protein level was significantly down-regulated in a concentration-dependent manner after treatment with curcumin. The WT1 mRNA levels were decreased by 20%, 25%, 40%, 78% and 88% in response to 10, 20, 30, 40 and 50 μM curcumin. The use of small inhibitory RNA (siRNA) targeting WT1 down-regulated the expression of WT1 about 90%. Combined treatment with curcumin and siRNA targeting WT1 resulted in a significant inhibition of cell proliferation compared to curcumin-treated cells alone. In conclusion, WT1 is involved in cellular proliferation of PANC-1 cells. Targeting WT1 gene expression with siRNA may enhance the efficacy of curcumin to inhibit cell proliferation.



## 1. Introduction

Despite pancreatic cancer being a rare cancer type with an incidence of 10–12 patients in 100,000 individuals in Europe and the United States of America (USA), it is the fourth leading cause of cancer death in Western countries. The overall survival rate of patients with pancreatic cancer, depending on the extent of disease and performance status (PS) at diagnosis, is extremely disappointing. At the time of diagnosis, approximately half of the patients have metastases, and their median survival does not exceed 6 months, whereas approximately one third of patients diagnosed with locally advanced disease have median survival times ranging between 6 and 9 months. Worldwide, more than 200,000 people die from pancreatic cancer each year due to the lack of sufficient systemic therapeutic options.[1–3]

Conventional chemotherapy has a limited effect on the course of disease progression in patients with pancreatic cancer, prompting a search for more effective agents. Therefore, the identification of molecular and pathophysiological underlying mechanisms in pancreatic cancer is essential.

Curcumin (diferuloylmethane) is a natural compound derived from the rhizome of *Curcuma longa*, also called turmeric. It has been shown that curcumin possesses potent

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anti-inflammatory and anti-oxidative properties, for which it has a long history of dietary use as a food additive. Curcumin modulates many intracellular signal transduction pathways, and has also a potent anti-proliferative activity against a variety of cancer cell lines *in vitro*.[4] Human phase I–II studies found curcumin to be safe, and indicated no dose-limiting toxicity when taken orally at doses up to 10 g/day. Recently, a phase II study was initiated with curcumin as a single agent in patients with pancreatic cancer demonstrating clinical efficacy and down-regulation of STAT3 in peripheral mononuclear cells.[5]

The Wilms' tumour gene (WT1) is located at chromosome 11p13. The WT1 protein contains a proline and glutamine-rich region mediating transcriptional regulation and four Cys2-His2-type zinc fingers.[6,7] The zinc finger domain binds to several DNA sequences.[8,9]

Curcumin has been shown to have anti-proliferative effects on leukaemic cells involving the Wilms tumour gene-1 (WT1) expression.[10–12] WT1 is over-expressed in a variety of malignancies, e.g. primary leukaemia,[13,14] lung cancer,[15] breast cancer,[16,17] colon cancer,[18] thyroid cancer[19], head and neck squamous cell cancer (HNSCC),[20] ovarian cancer[21] and pancreatic cancer[22–24] as well as bone and soft-tissue sarcoma.[25] The growth of WT1-expressing cancer cells can be inhibited by an exposure to Ribozymes, antisense oligonucleotides (ASOs) or siRNA targeting WT1.[22,26–31]

Over the past three decades, studies dealing with the absorption, distribution, metabolism and excretion of curcumin have revealed poor absorption and rapid metabolism of curcumin leading to severe reduced bioavailability. Low serum levels, limited tissue distribution, apparent rapid metabolism and short half-life are the limiting factors for curcumin administration in human cancers.[32] On the other hand, a major advantage of curcumin is the interaction with multiple cancer relevant proteins and signalling pathways.[4]

The aim of our study was to investigate the role of WT1 in the inhibition of cellular proliferation after curcumin treatment. Therefore, we have silenced the WT1 gene expression with siRNA prior to treatment with curcumin.

## 2. Materials and methods

### 2.1. Cell culture

The pancreatic cancer cell line PANC-1 (kindly provided by PD Dr. F. Gansauge, Ulm, Germany) was maintained in RPMI 1640 supplemented with 10% heat inactivated foetal calf serum. The cell line was tested negative for mycoplasma using the PCR-mycoplasma diagnostic-kit VenorGeM (Minerva biolabs, Berlin, Germany). Foetal calf serum (FCS) was purchased from PAA Laboratories (Coelbe, Germany). RPMI-1640, phosphate-buffered saline (PBS), Opti-MEM I, Lipofectamine2000 and glutamine were obtained from Invitrogen (Karlsruhe, Germany).

### 2.2. siRNA transfection

siRNA was synthesised by MWG, Ebersberg, Germany. siRNA sequence targeting wt1 (National Center for Biotechnology Information [NCBI] accession number AH003034) was synthesised. GGACUGUGAACGAAGGUUU corresponds to positions 28–46 within exon 8 of the WT1 open reading frame. The WT1 siRNA sequence was subjected to BLASTN 2.2.9-program to avoid homologue human sequences of other genes. Unspecific control siRNA was obtained from Santa Cruz (sc-37007). The cells were transfected with siRNA using the Lipofectamine™2000 protocol (Invitrogen, Life Technologies). In brief, 1 day prior to transfection cancer cells were seeded out at a concentration of $1 \times 10^5$ cells per 25 $cm^2$ flask. Briefly, $10^6$ cells in a 75 $cm^2$ flask were transfected with 2.5 μg of WT1 siRNA or control siRNA diluted in 2 ml of OPTI-MEM (Invitrogen Life Technologies) and 10 μl/ml Lipofectamine 2000. After a 4 h incubation of cells, the medium was replaced with RPMI-1640 Medium (PAA, Coelbe, Germany). In some experiments after 24 h, the cells were incubated with different concentrations of curcumin for mRNA analysis, protein expression and FACS analysis. All transfections were performed in triplicate for each time point.

### 2.3. Drugs and antibodies

Curcumin (min. 94%) was purchased from Sigma–Aldrich. Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Loughborough, UK). A 10 mM curcumin stock solution was dissolved in DMSO and stored at –20 °C until use. Monoclonal anti-human WT1 (F-6), anti-STAT3 (F-2), anti-pSTAT3 *tyr705* (B-7), anti-PARP-1 (F-2), anti-α-Tubulin (TU-02) and goat anti-mouse secondary antibody for Western blot were obtained from Santa Cruz Biotechnology, Inc. The antibody anti-pSTAT3 *ser727* was obtained from CellSignaling.

### 2.4. Proliferation assay (MTT-assay)

Cell proliferation was assessed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma–Aldrich, Deisenhofen, Germany). Cells were incubated with 2.5 μg siRNA and different concentrations of curcumin and curcumin/WT1 siRNA, respectively, for 24, 48 and 72 h. All assays were performed in triplicate. Then a solution of MTT in phosphate-buffered saline (PBS) was added to each well to a final concentration of 5 mg/ml. After 3 h incubation in the dark, blue formazan was solubilised with 2000 μl isopropanol/0.04 N HCl. Absorbance was measured at 590 nm and 690 nm using a photometer.

### 2.5. RNA preparation and RT-PCR

Cells were harvested, centrifuged and washed with PBS. Total RNA was isolated with RNeasy Mini kit according to manufacturers' protocol (Qiagen, Hilden, Germany). All cDNA products were purchased from (Invitrogen, Karlsruhe, Germany). The cDNA was synthesised from 2 μg total RNA and 1 μl (200 U) Superscript II RT, 4 μl 5 × first strand buffer, 1 μl random primers, 2 μl DTT (0.1 mM), 0.1 μl dNTP-mix (100 mM) in 20 μl reaction volume.

Reaction conditions were 25 °C 10′, 42 °C 50′ and 70 °C 5′. The following primers were used:

| | |
|---|---|
| WT1 | up 5′-GGCATCTGAGACCAGTGAGAA-3′, down 5′-TCAAAGCGCCAGCTGGAGTTT-3′, 857 bp |
| GAPDH | up 5′-GCAGGGGGAGCCAAAAGG-3′ down 5'-TGCCAGCCCCAGCGTCAAAG-3', 566 bp |

2 μl of cDNA was subjected to PCR amplification in a Biometra Thermocycler. PCR conditions were 5 μl 10 × PCR buffer containing 3 μl $MgCl_2$ (1.5 mM), 1 μl dNTP (0.2 mM), 0.5 μl up and down Primer (100 pM) and 0.3 μl Hot Start Taq-Polymerase (ABgene) in 50 μl final volume. An initial cycle was performed at 95 °C for 15 min, following 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 40 s and extension at 72 °C for 1 min. The PCR products were visualised after electrophoresis with 1.5% agarose gel containing 1 μl ethidium bromide (1 mg/ml) per 100 ml agarose.

### 2.6. Real-time RT-PCR

The cDNA was subjected to quantitative real-time PCR analyses targeting WT1 and GAPDH. Analyses were performed using the StepOne Real-Time PCR System and the StepOne v2.0 software (Applied Biosystems). Relative gene expression values were determined by the ΔΔCT method using the StepOne v2.0 software (Applied Biosystems). Data are presented as the fold difference in WT1 (Hs00240913_m1) expression normalised to the housekeeping gene GAPDH (4333764F) as an endogenous reference, and relative to the untreated control cells. Amplification mixes (20 μl) contained 1 μl of 20X TaqMan Gene Expression Assay Mix (Applied Biosystems), 2 μl cDNA template, 10.0 μl ABsolute QPCR ROX Mix (2X) (ABgene) and 7 μl Rnase-free water. Thermal cycling consisted of 15 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s using the StepOne System (AppliedBiosystems). All tests were carried out in triplicate.

### 2.7. FACS analysis (Becton Dickinson FACS)

The annexin V FACSAnalysis was performed according to the manufacturers' protocol (Annexin V Fluorescein In situ apoptosis detection kit, R&D Systems). Approximately $1 \times 10^4$ cells/well were plated in a 48-well tissue culture plate (Becton Dickinson, NJ, USA). Cells were incubated with curcumin at various concentrations (0–50 μM). Cells were washed with PBS and resuspended in 100 μl annexin V incubation buffer and incubated for 15 min at room temperature in the dark. 400 μl of 1× binding buffer to each sample was added and analysed with flow cytometry. The fluorescence of 10,000 cells was determined after subtracting the background fluorescence of control cells.

### 2.8. Western blot analysis

For Western blot analysis, the cells were harvested in lysis buffer (0.05 M TrisHCl, pH 6.8, 2% SDS). The viscosity was reduced by incubation with benzonase (Merck, Darmstadt, Germany). The homogenate was centrifuged for 10 min at 14,000*g*, and the supernatant was taken for protein determination. Cell lysates (50 μg) were denatured in Laemmli buffer at 95 °C, and loaded on 10% SDS gel, separated at 100 V for 2 h and electrophoretically transferred to Hybond-C membrane (Amersham Pharmacia Biotech). The membranes were incubated for 1 h in 2% powdered non-fat milk, 0.05% Tween-20 in PBS with monoclonal antibodies anti-WT1, and anti-α-Tubulin (1:1000). The membranes were then incubated for 30 min in 2% powdered non-fat dry milk, 0.05% Tween-20 with goat anti-mouse serum (1:2000). Bound antibody was detected by chemiluminescence using ECL reagents (Amersham Pharmacia Biotech).

### 2.9. Statistical analysis

All data are expressed as mean ± SD from triplicate samples of three independent experiments.

## 3. Results

### 3.1. The effect of curcumin on cell proliferation

Curcumin inhibits the proliferation of the pancreatic cancer cell line PANC-1 in a concentration-dependent manner (Fig. 1).

### 3.2. The effect of curcumin on WT1 mRNA expression

PANC-1 is a WT1 positive cell line expressing WT1 mRNA as well as WT1 protein (Fig. 2 and 5F).

### 3.3. Real-time RT-PCR evaluation of WT1 mRNA down-regulation

To determine the effect of curcumin on WT1 mRNA expression and protein level, PANC-1 cells were incubated with different amounts of curcumin for 24 h. The WT1 mRNA levels were decreased by 20%, 25%, 40%, 78% and 88% in response to 10, 20, 30, 40 and 50 μM curcumin as determined with real-time RT-PCR (Fig. 3).

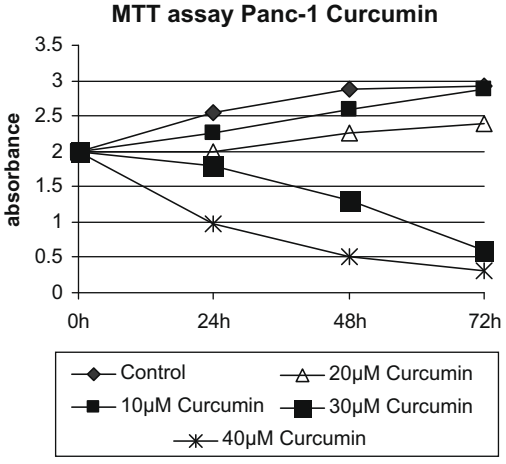


**Fig. 1 – The incubation of PANC-1 cells with various concentrations of curcumin (0–40 μM) resulted in a concentration-depending inhibition of cell proliferation (Fig. 1).**

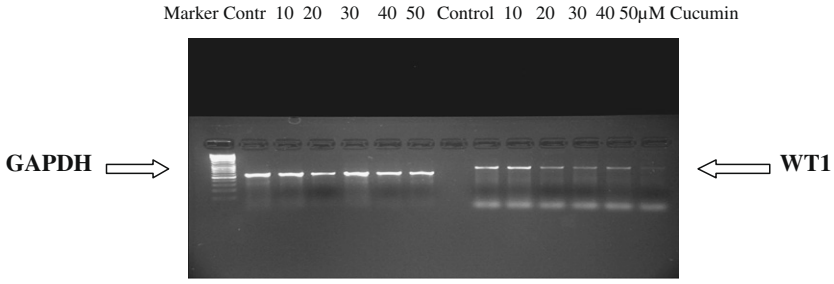


**Fig. 2 – The conventional RT-PCR mRNA indicates a nearly concentration-depending down-regulation of WT1 mRNA after treatment of PANC-1 cells with curcumin. GAPDH served as a housekeeping gene.**

### 3.4. *The effect of combined WT1 siRNA and curcumin treatment on the WT1 mRNA level*

To evaluate the effect of WT1 siRNA on the expression of WT1 mRNA in PANC-1, cells were seeded into 75 $cm^2$ flasks and cultivated until 60% confluency. The effect of 10 μM curcumin was about 20% decrease of WT1 mRNA after 24 h. The treatment of PANC-1 cells with WT1 siRNA resulted in a down-regulation of WT1 mRNA expression by 90% after 24 h as determined with real-time RT-PCR. The combination of WT1 siRNA and curcumin resulted in a marginal decrease of WT1 mRNA compared to WT1 siRNA alone (Fig. 4).

### 3.5. *The effect of curcumin on protein levels*

The effect of curcumin on different proteins was detected with Western blot. The PANC-1 cells were incubated with different amounts of curcumin (0–50 μM) over a period of 48 h.

The analysis of PARP-1 protein by Western blot did not show any 85 kDa cleavage products indicating induction of apoptosis (Fig 5B). This is in line with our AnnexinV/PI-FACS-Analysis, where no apoptosis was detected (data not shown).

The expression of STAT3 is nearly unaffected even after incubation with 50 μM curcumin (Fig. 5C). The expression of pSTAT3*ser727* is not affected either (Fig. 5D). But curcumin down-regulated selectively pSTAT3*tyr705* in PANC-1 cells in a dose-dependent manner (Fig. 5E). Interestingly, this dose-dependent down-regulation of pSTAT3tyr705 is reflected in the down-regulation of WT1 as shown in Fig. 5F. We only can speculate if pSTAT3*tyr705* may be involved in the regulation of WT1 gene expression.

### 3.6. *The MTT assay to evaluate the effect of WT1 down-regulation prior to curcumin treatment*

These results implicate a role of WT1 in curcumin-resulted inhibition of cell proliferation. Therefore, we have incubated PANC-1 cells with siRNA targeting WT1 24 h before incubation with different concentrations of curcumin. The down-regulation of WT1 prior to treatment with curcumin resulted in a much higher inhibition of cell proliferation compared to the incubation with curcumin alone (Fig. 6).

## 4. Discussion

Pancreatic cancer is the fourth most common cause of cancer-related death in the Western world with very limited therapeutic options.[33] There is a need for new therapeutic

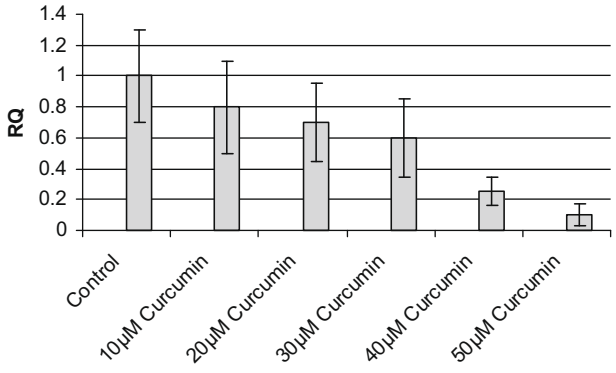


**Fig. 3 – The real-time RT-PCR shows the relative expression of the WT1 mRNA after treatment with curcumin for 24 h. The expression level is significantly down-regulated after treatment with concentrations more than 30 μM curcumin. The WT1 mRNA levels were decreased by 20%, 25%, 40%, 78% and 88% in response to 10, 20, 30, 40 and 50 μM curcumin.**

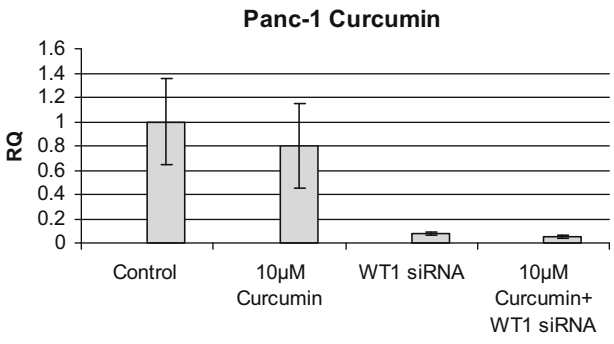


**Fig. 4 – The figure shows the relative expression of WT1 mRNA after treatment with DMSO (control cells), 10 μM curcumin, WT1 siRNA alone or as a combination of WT1 siRNA and 10 μM curcumin after 24 h. The WT1 siRNA down-regulates the expression of WT1 significantly about 80–90% compared to control cells. Adjacent incubation with 10 μM curcumin resulted only in a marginal further down-regulation.**

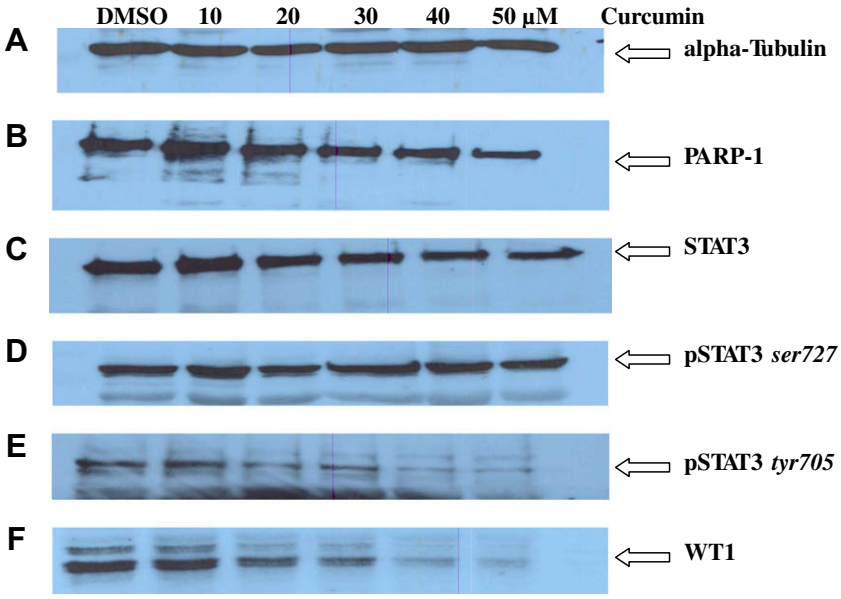


**Fig. 5 – To evaluate the effect of curcumin on the expression of various proteins, PANC-1 cells were treated with DMSO or 10, 20, 30, 40 and 50 μM curcumin for 48 h. Whole cell-lysates were analszed by Western blot as described in Materials and Methods. (A) alpha–Tubulin shows equal loading. (B) Expression of PARP-1 shows no 85 kDa cleavage product indicating apoptosis. (C) The expression of STAT3 and (D) pSTAT3*ser727* is nearly unaffected. (E) The down-regulation of pSTAT3*tyr705* correlates with the expression of WT1 (F).**

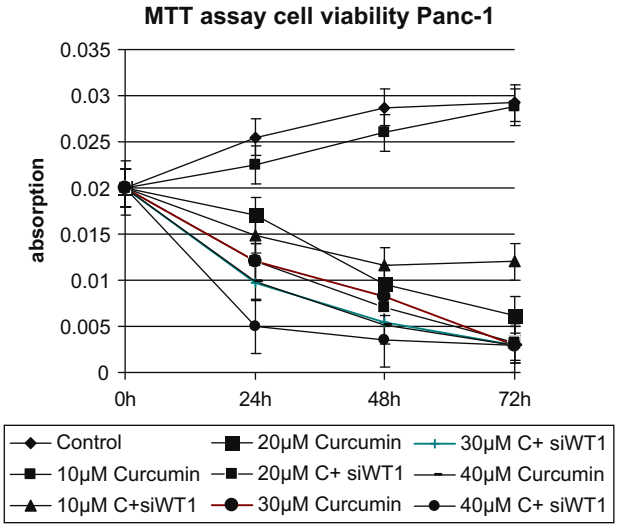


**Fig. 6 – The supporting effect of WT1 down-regulation prior to curcumin treatment is shown. The MTT assay shows the inhibition of cell proliferation of PANC-1 cells. The cells (60% confluency) were treated with control siRNA (control), curcumin (10–40 μM) or curcumin (10–40 μM) + WT1 siRNA (2.5 μg) over a period of three days. The silencing of WT1 gene expression with adjacent curcumin resulted in a significant inhibition of cell proliferation compared to treatment with curcumin alone. WT1 modulation enhanced the curcumin sensitivity corresponding to about 10 μM higher curcumin concentrations. Error bars represent the standard error of the mean.**

strategies. WT1 has been identified as a potential molecular target for cancer therapy. In pancreatic cancer, immunohistochemical analysis revealed 35–65% WT1 positive cases.[23] PANC-1 cells express high levels of WT1. The expression level of WT1 of our PANC-1 cell line is about 60% higher compared to K562 cells as determined with real-time RT-PCR (own results, data not shown). After incubation with curcumin (0–50 μM), the expression of WT1 mRNA and WT1 protein level was significantly down-regulated mainly at high concentrations between 30 and 50 μM. Those concentrations, however, are hardly achievable in the serum of human patients. The down-regulation of WT1 gene expression implicated a significant role in curcumin-dependent inhibition of cell proliferation. To increase the sensitivity of curcumin to the pancreatic cancer cells, we inhibited WT1 expression with siRNA prior to incubation with curcumin.

PANC-1 is a widely chemoresistant pancreatic cancer cell line including gemcitabine and bortezomib[34], but curcumin is able to inhibit the proliferation in PANC-1 in a dose-and time-dependent manner. The down-regulation of WT1 with siRNA prior to incubation with curcumin resulted in a dramatic inhibition of cell proliferation even at low concentrations between 10 and 20 μM curcumin. Despite other reports on apoptosis induced by curcumin, we were not able to detect any significant induction of apoptosis measured with either Annexin V/PI-staining FACSAnalysis or PARP-1 cleavage products analysed with Western blotting.[35] WT1 modulation enhanced the curcumin sensitivity corresponding to about 10 μM higher curcumin concentrations. So, WT1 may serve as a marker for curcumin sensitivity in individual patients. A corresponding relationship was shown by the group of Cilloni[36], who predicted the sensitivity of Imatinib therapy by

the ability to inhibit WT1 gene expression in patients with chronic myelogenous leukaemia (CML).

Rong et al.[37] described a synergistic role for signal transducers and activators of transcription 3 (STAT3) and WT1 in tumour development and raised the hypothesis that WT1 and activated STAT3 might accelerate tumourigenesis. Signal transducer and activator of transcription 3 (STAT3) protein is a member of a family of latent cytoplasmic transcription factors transmitting signals from the cell surface to the nucleus activated by cytokines and growth factors. STAT3 phosphorylation plays a critical role in transformation and proliferation in a variety of tumour cells e.g. breast, lymphoid and myeloid cells.[38] Numerous studies have detected constitutively active STAT3 in diverse human tumour specimens and established persistent STAT3 activity as essential for malignant transformation of cultured cells influencing many oncogenic signalling pathways.

In regard to possible interactions between WT1 and STAT3, it was of interest to investigate the effect of curcumin on STAT3 phosphorylation in PANC-1 cancer cell lines. The activation of STAT3 occurs through tyrosine phosphorylation at a single tyrosine residue (*tyr705*) allowing STAT dimerisation and transcription activation.[39] For maximal transcriptional activity both residues (*tyr705* and *ser727*) have to be phosphorylated.[40,41] Our results show that curcumin eliminated the constitutively phosphorylated form of STAT3*tyr705* in a concentration-dependent manner indicating a possible interaction with WT1. The phosphorylation of STAT3*ser727* was not inhibited. STAT3 plays a key role in G1- to S-phase cell cycle transition.[42] The proliferation assay revealed this blockade of cell proliferation. Interestingly, a down-regulation of STAT3*tyr705* was also seen in the peripheral blood mononuclear cells (PBMCs) from patients suffering from pancreatic cancer treated with curcumin.[5]

We were able to demonstrate that curcumin down-regulates the gene expression of WT1 on mRNA and protein level. There is considerable evidence that WT1 is involved in the transformation of cells, suggesting WT1 as a possible anticancer target.

In conclusion, our study indicates that the down-regulation of WT1 mediated by siRNA could inhibit the growth of pancreatic cancer cells and may enhance its sensitivity to curcumin. We consider that siRNA against WT1 in combination with curcumin might be of potential value for the treatment of human pancreatic cancer.

## Conflict of interest statement

On the behalf of the authors I indicate that none of the authors have any financial disclose or any personal relationships with other people or organisations that could inappropriately influence (bias) this work.

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