

RESEARCH ARTICLE

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Wilms' tumor gene 1 regulates *p63* and promotes cell proliferation in squamous cell carcinoma of the head and neck

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Abstract

Background: Wilms' tumor gene 1 (*WT1*) can act as a suppressor or activator of tumorigenesis in different types of human malignancies. The role of *WT1* in squamous cell carcinoma of the head and neck (SCCHN) is not clear. Overexpression of *WT1* has been reported in SCCHN, suggesting a possible oncogenic role for *WT1*. In the present study we aimed at investigating the function of *WT1* and its previously identified protein partners *p63* and *p53* in the SCCHN cell line FaDu.

Methods: Silencing RNA (siRNA) technology was applied to knockdown of *WT1*, *p63* and *p53* in FaDu cells. Cell proliferation was detected using MTT assay. Chromatin immunoprecipitation (ChIP)/PCR analysis was performed to confirm the effect of *WT1* on the *p63* promoter. Protein co-immunoprecipitation (co-IP) was used to find protein interaction between *WT1* and *p53/p63*. Microarray analysis was used to identify changes of gene expression in response to knockdown of either *WT1* or *p63*. *WT1* RNA level was detected using real-time quantitative PCR (RT-qPCR) in patients with SCCHN.

Results: We found that *WT1* and *p63* promoted cell proliferation, while mutant *p53* (R248L) possessed the ability to suppress cell proliferation. We reported a novel positive correlation between *WT1* and *p63* expression. Subsequently, *p63* was identified as a *WT1* target gene. Furthermore, expression of 18 genes involved in cell proliferation, cell cycle regulation and DNA replication was significantly altered by downregulation of *WT1* and *p63* expression. Several known *WT1* and *p63* target genes were affected by *WT1* knockdown. Protein interaction was demonstrated between *WT1* and *p53* but not between *WT1* and *p63*. Additionally, high *WT1* mRNA levels were detected in SCCHN patient samples.

Conclusions: Our findings suggest that *WT1* and *p63* act as oncogenes in SCCHN, affecting multiple genes involved in cancer cell growth.

Keywords: *WT1*, *p63*, *p53*, Cell proliferation, Squamous cell carcinoma of the head and neck (SCCHN)

Background

Squamous cell carcinoma of the head and neck (SCCHN) is the sixth most common cancer and also the most common tumor type in the head and neck region. The 5-year survival is approximately 50% and has increased only marginally during the last decades. The molecular pathogenesis of SCCHN is not yet completely understood, a fact that complicates development of new therapeutic approaches [1]. Mutations in the *p53* gene have been

reported in one to two thirds of SCCHN [2]. The *p53*-related transcription factor, *p63*, is reported to be overexpressed in the majority of primary SCCHN tumors [3,4]. *p63* expression is regulated through two distinct promoters, giving rise to two main isoforms, TAp63 and ΔNp63. TAp63 is transcribed from the external promoter which contains the transactivating domain homologous to *p53*, enabling it to regulate transcription of *p53* target genes. ΔNp63 is transcribed from an internal promoter and acts in a dominant negative fashion with the ability to overcome the cell cycle arrest and apoptosis normally driven by *p53* [5]. The main isoform overexpressed in SCCHN is ΔNp63α, a critical pro-survival protein [6,7].

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Wilms' tumor gene 1 (*WT1*) was first identified as a tumor suppressor gene in Wilms' tumor, a childhood kidney neoplasm [8]; later findings demonstrated oncogenic properties in other malignancies including breast [9], lung [10,11], ovarian [12,13] and brain tissue [14]. *WT1* was previously found to interact with p53 and p63 at protein level in baby rat kidney cells and in Saos-2, an osteosarcoma cell line [15,16]. However, the interaction has not been studied in any other cell types yet.

In SCCHN, *WT1* overexpression has been reported by Oji et al. [17] suggesting an oncogenic property. However, no functional study has been performed to investigate the role of *WT1* in SCCHN tumorigenesis.

In the present study, our aims were to investigate the function of *WT1* in SCCHN and to examine possible interactions between *WT1* and p63/p53. A positive correlation between *WT1* and p63 was found in FaDu cells, an SCCHN cell line. CHIP analysis verified *WT1* binding to the *p63* promoters, designating *p63* a target gene of *WT1*. The functional link between *WT1* and *p63* was further demonstrated by altered expression of several known p63 target genes in *WT1* knockdown cells. By silencing *WT1* and *p63* RNA, SCCHN cell proliferation was decreased. *WT1* and p63 were found to generate effects on cell proliferation through multiple genes involved in cell proliferation, cell cycle regulation and DNA replication.

Methods

Cell culture

The FaDu cell line (ATCC HTB-43), derived from hypopharyngeal squamous cell carcinoma, was used for transfection experiments. The cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Stockholm, Sweden) containing 10% fetal bovine serum (Gibco) in 5% CO₂ at 37°C.

siRNA and *WT1D* plasmid transfection

Pooled siGENOME SMART pool of *WT1*, *p63* and *p53* siRNA (Dharmacon, Chicago, USA) was used for transfection. To suppress expression of *WT1*, *p63* and *p53*, FaDu cells were transiently transfected with siRNA of *WT1* (12.5 nM/well), *p63* (5 nM/well) and *p53* (5 nM/well) in six well plates (3 × 10⁵ cells/well) and 96-well plates (8 × 10³ cells/well). Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) was used for suppression of gene expression. Cells were harvested at 24, 48 or 72 hours after transfection for further analysis. To induce *WT1D* overexpression, pcDNA 3.1 (+) vectors (Invitrogen, Carlsbad, CA, USA) ligated with *WT1* variant D were constructed as previously described [18]. FaDu cells were transiently transfected with 3 μg *WT1D* pcDNA 3.1 (+) vectors per well in six-well plates (5 × 10⁵ cells/well) using lipofectamine 2000 (Invitrogen).

MTT assay

Vybrant MTT Cell Proliferation Assay Kit (Invitrogen) was applied to measure cell proliferation. FaDu cells were collected at 0, 24 and 48 hours after transfection and labeled with MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) mixed with SDS-HCL. Absorbance was measured on spectrometer at 570 nm wavelength.

Western blot

Total protein was extracted using lysis buffer (0.5% NP-40, 0.5% NA-DOC, 0.1% SDS, 150nM NaCl, 50 mM Tris pH 7.5, 1 mM EDTA, 1 mM NaF) supplemented with protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA). Protein concentration was measured using BCA reagent (Thermo Scientific, Rockford, IL, USA). Twenty μg of each sample was separated using 10% SDS polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA, USA) and then transferred to a PVDF membrane (Millipore, Billerica, MA, USA). The membrane was blocked using TBST containing 5% non-fat dry milk, then incubated with mouse-monoclonal antibodies against *WT1* (1:250, catalog no. M3561, DAKO, Glostrup, Denmark), p63 (1:2000, catalog no. M7247, DAKO), p53 (1:1000, catalog no. PAb 1801, Abcam, Cambridge, UK) and β-actin (1:10000, catalog no. MAB1501R, Millipore) followed by a second incubation with peroxidase conjugated anti-mouse polyclonal antibodies (1:5000, DAKO). The antibody (anti-p63) used in this study is able to detect bands corresponding to the expected molecular weights and according to expression patterns of the various isoforms (TAp63α, TAp63γ, ΔNp63α, and ΔNp63γ). Proteins were visualized using a chemiluminescent detection system (ECL-advanced, GE healthcare UK) in ChemiDoc XRS (Bio-Rad, Italy).

RNA extraction and cDNA preparation

Total RNA was extracted using TRIzol reagent (Invitrogen, Stockholm, Sweden). cDNA was prepared using superscript II reverse transcriptase kit according to the manufacturer's instructions (Invitrogen).

Chromatin immunoprecipitation (ChIP)/PCR analysis

ChIP analysis was performed using the Chromatin Immunoprecipitation Kit (Upstate Millipore, Billerica, MA, USA). SKOV-3 cell line, derived from the ascitic fluid of a female with an ovarian tumor (ATCC HTB-77) with no endogenous *WT1* expression and null p53 expression (p53 mutation at codon 89 and 179) was used as an extra negative control [19,20]. Approximately 1 × 10⁶ FaDu cells with or without *WT1D* transfection and SKOV-3 cells were crosslinked with 1% formaldehyde, followed by glycine to quench unreacted formaldehyde. Chromatin was sonicated on ice to shear crosslinked DNA to about 200–1000 bp in length using a sonifier ultrasonic cell disrupter (Branson, Danbury, CT,

USA) with 12×10 s pulses. The sheared chromatin was re-suspended in dilution buffer and 1% of the chromatin was removed as input, followed by immunoprecipitation using protein G magnetic beads with 2 μ g of either anti-WT1 (C-19) antibody (catalog no. sc-192, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) or normal rabbit IgG (catalog no. 2729S, Cell Signalling technology Inc, Danvers, MA, USA) at 4°C overnight with rotation. After the reversal of crosslinks by incubation in ChIP elution buffer containing proteinase K at 62°C for 2 h, DNA was purified using spin columns. PCR reactions containing 2 μ l of the immunoprecipitated DNA or input chromatin, primers and AmpliTaq Gold (Applied Biosystem) in a 25 μ l volume were performed with initial denaturation at 95°C for 10 min, followed by 35 cycles (95°C for 30 s, 60°C for 30s and 72°C for 45 s) and a final extension at 72°C for 10 min. Primer sequences for *p63* promoters are shown in Additional file 1: Table S1. PCR products were fractionated on 1% agarose gel and ethidium bromide stained DNA was visualized on Ultraviolet Transilluminator (Spectroline, Westbury, NY, USA). For quantitative real-time PCR, SYBR green master mix (Bio-Rad) was used in a 25 μ l volume of reaction. For PCR amplification of cDNA, IQ Sybr Green supermix (Bio-Rad) was used, and samples were analyzed on Iq5 (Bio-Rad). The primer sequences are the same as the sequences listed in Additional file 1: Table S1.

Genome-wide gene expression array

From each sample, 200 ng RNA was used to produce biotinylated cRNA using TargetAmp-Nano labeling kit (Illumina, San Diego, CA, USA). A total of 750 ng biotinylated cRNA was hybridized to an Illumina HumanHT-12 v4 Expression BeadChip according to the manufacturers' protocol (Illumina). Arrays were scanned using Illumina iScan Reader. The GenomeStudio (Illumina) software was used for data processing. For normalization, background correction and variance stabilization transformation Lumi package was used [21]. Differentially expressed genes were identified based on a moderated *t* test using MEV software package from TIGR [22]. Network analysis was carried out with the Metacore software (GeneGo Inc, St Joseph, MI, USA). Pathway analysis was carried out using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) tool [23].

Protein co-immunoprecipitation (co-IP)

FaDu cells were lysed in cold lysis buffer (0.5% NP-40, 0.5% NA-DOC, 0.1% SDS, 150nM NaCl, 50 mM Tris pH 7.5, 1 mM EDTA, 1 mM NaF) supplemented with protease inhibitor (Sigma-Aldrich, St. Louis, USA) for 30 min at 4°C; lysates were clarified by centrifugation at 14,000 rpm for 30 min at 4°C. Equivalent amounts of protein lysate were incubated with the anti-WT1 (catalog no. M3561, DAKO, Glostrup, Denmark), anti-IgG (catalog no.

2729S, Millipore, Billerica, U.S.A.) antibodies at 4°C overnight, then incubated with Protein G Sepharose 4 Fast Flow (GE Healthcare, Uppsala, Sweden) at 4°C for 1 hr. Immunoprecipitates were washed with lysis buffer three times. Immunoprecipitated proteins were eluted with SDS-sample buffer and analyzed by SDS-PAGE and Western blotting. Immuno-blotting was conducted using anti-WT1 (1:250, catalog no. M3561, DAKO, Glostrup, Denmark), p53 (1:2000, catalog no. PAb 1801, Abcam, Cambridge, UK) and p63 (1:2000, catalog no. M7247, DAKO, Glostrup, Denmark).

Patient samples and real-time quantitative PCR

After obtaining informed written consent, tumor biopsies were taken from 15 patients with SCCHN, clinically adjacent tumor-free tissue was available from 7 of the patients. Punch biopsies were taken from 14 healthy non-smoking volunteers. The tissue specimen collection had been approved by the Ethics Committee at Umeå University (Dnr 01–057). *WT1* mRNA level was quantified by real-time quantitative PCR (RT-qPCR) using TaqMan technology in 7900HT system (Applied Biosystems, Foster City, CA, USA). RT-qPCR reactions were carried out in a 25 μ L volume containing 12.5 μ L universal PCR master mix, each primer at a concentration of 0.5 mM, probe at 0.1 mM, and 50 ng of cDNA. Triplicate assays were run in parallel for each sample. *WT1* transcription values were normalized against the expression of β -*actin*, to adjust for variations in RNA and cDNA synthesis. The mean of triplicates of the *WT1* gene copy numbers was divided by the mean of duplicates of copy numbers of the β -*actin*. Primers and probes for the *WT1* and β -*actin* gene and the amplification conditions have been described previously [24].

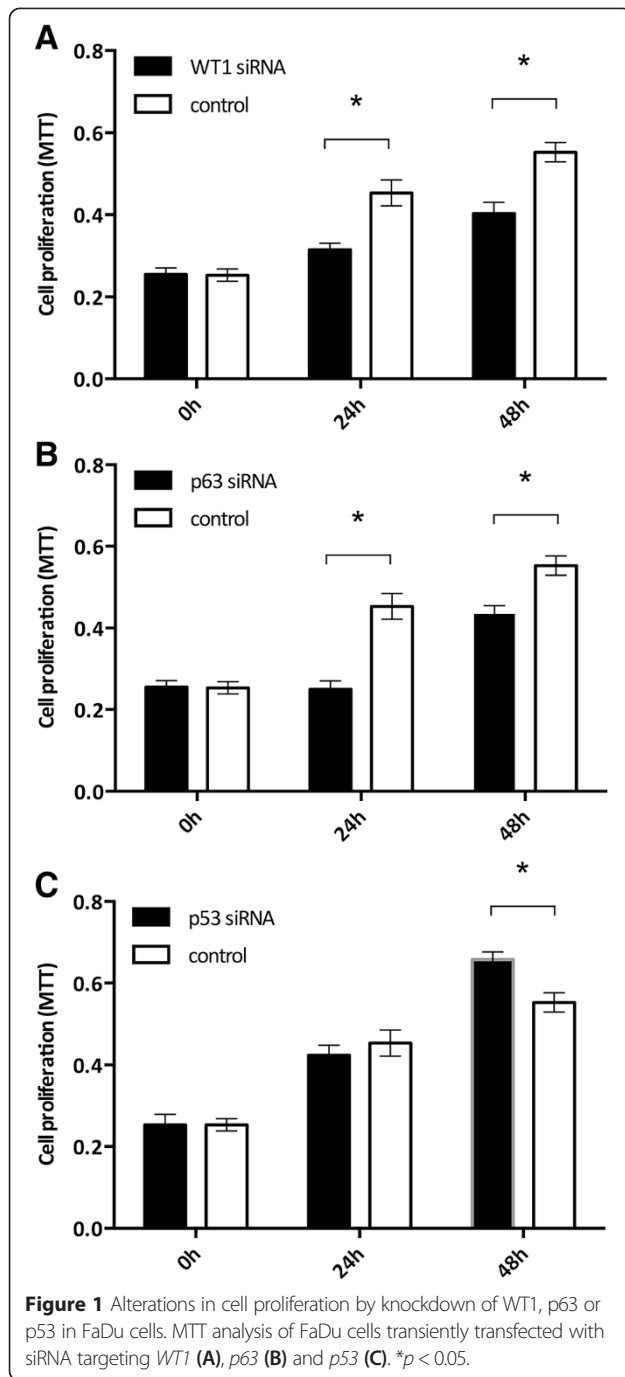
Statistical analysis

Statistical analysis was performed using SPSS (version 19, SPSS Inc., Chicago, IL, USA). Mann–Whitney *U*-test was used to compare differences in the expression of two different variables. Fisher's exact tests (when sample size was <5) were used for comparison of proportions. A *p*-value < 0.05 was considered to be significant.

Results

Altered cell proliferation through knockdown of WT1, p63 and p53

To determine the effect of WT1, p63 and p53 on cell proliferation in FaDu cells *in vitro*, MTT assays were performed. Knockdown of *WT1* resulted in a significant decrease in cell proliferation at 24 and 48 hours after transfection (*p* < 0.05, Figure 1A). Similarly, silencing *p63* RNA induced a considerable decrease in cell proliferation at both time points (*p* < 0.05, Figure 1B). These



results indicate that both *WT1* and *p63* have a positive effect on cell proliferation in FaDu cells.

p53 function is inactivated in up to 80% of HNSCC [25]. In the FaDu cell line, *p53* has a point mutation at codon 248 (Arg → Leu) [26]. The R248L mutation of *p53* does not completely abolish its inhibitory effect on cell proliferation in this cell line. As shown in Figure 1C, a significant increase in cell proliferation in *p53* knockdown cells was demonstrated at 48 hours after transfection compared to control cells ($p < 0.05$).

Correlation between *WT1* expression and *p63/p53* in FaDu cells

Previous studies have demonstrated a protein-protein interaction between *WT1* and *p63/p53* [16,27]. Furthermore, *WT1* has been reported to exert protein stabilization on *p53* in some cellular settings [15]. In order to study the relationship between *WT1* and *p63/p53* in SCCHN, transfection experiments in FaDu cells were performed. Suppressed expression of *WT1*, *p63* and *p53* were induced using siRNA technologies.

Successful silencing of *WT1* RNA resulted in downregulated expression of *WT1* protein as seen on western blot (Figure 2A). Distinctly decreased expression of Δ Np63 (68 kDa) was observed in cells with suppressed *WT1* expression compared to control cells. However, we found that expression of the TAp63 α (75 kDa) was much weaker than Δ Np63 α (68 kDa). TAp63 α showed no changes in expression in our experiments. γ -isoforms (TAp63 γ or Δ Np63 γ) were not detectable in FaDu cells (data not shown). A slight decrease in protein expression of *p53* in *WT1* knockdown cells was observed only at 72 hours after transfection.

Knockdown of *p63* induced a slight decrease in protein expression of *WT1* at 48 and 72 hours after transfection (Figure 2B). Decreased expression of *p53* was observed only at 72 hours after transfection.

No alterations of *WT1* or *p63* protein expression were observed in *p53* knockdown cells (Figure 2C).

An additional experiment was performed to confirm the positive correlation between *WT1* and Δ Np63 using a plasmid carrying *WT1D* variant into FaDu cells. Up-regulation of Δ Np63 protein levels was observed in cells with forced overexpression of *WT1D* (Figure 2D). Again, altered expression of TAp63 α was not found (data not shown).

These results indicate a possible functional link between *WT1* and *p63* in FaDu cells, but not a strong association between *WT1* and *p53* expression.

p63 is a *WT1* target gene

A positive correlation between *WT1* and *p63* gene expression was found as described above. To assess whether *p63* is a target gene of *WT1*, the binding properties of *WT1* to the *p63* promoters were examined using ChIP/PCR. Two putative GNGNGGGNG *WT1*-binding sites in the TAp63 promoter and one putative *WT1*-binding site in the Δ Np63 promoter were identified by sequencing analysis (Additional file 1: Table S1). ChIP was performed with *WT1D* transfected and non-transfected FaDu cells and chromatin precipitated with *WT1* antibodies. PCR amplification products could be demonstrated in the region of the second *WT1*-binding site of the TAp63 promoter and at the Δ Np63 *WT1*-binding site (Figure 3A). Results were also confirmed with quantitative real-time PCR (Figure 3B

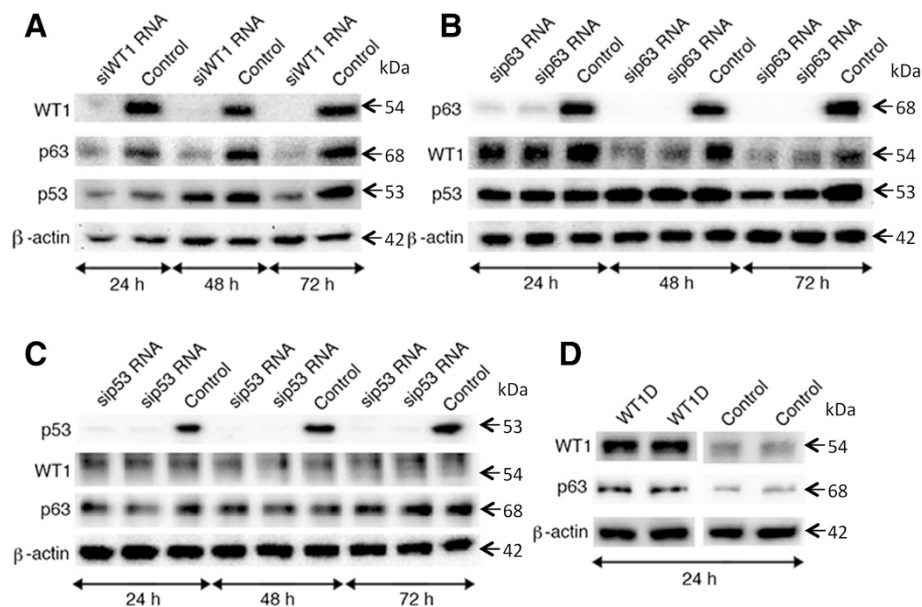


Figure 2 Alterations of protein expression of WT1 and p63/p53 using *in vitro* experiments in FaDu cells, demonstrated by western blot. Cells were harvested at 24, 48 or 72 hours after transient transfection with siRNA targeting WT1 (A) p63 (B) p53 (C) and after WT1D plasmid transfection at 24 hours (D).

for the TAp63 second binding site and Figure 3C for the $\Delta Np63$ WT1-binding site). Consequently, using ChIP/PCR assay we could demonstrate direct binding of WT1 to the p63 promoters.

WT1 can regulate p63 transcription through multiple genes involved in cell growth

Genes with altered expression in response to knockdown of WT1 or p63 were detected with microarray analysis. Silencing WT1 RNA induced significant fold changes of 848 genes compared to control (Figure 4A). Significantly altered expression of 925 genes was found in cells with suppressed p63 expression. Interestingly, by combining the two profiles we found that 124 genes had significantly altered fold changes ($p < 0.05$, Figure 4A). Eighteen of these genes were found to be involved in cell proliferation, cell cycle regulation and DNA replication (Table 1). Ten genes involved in cell proliferation, five genes involved in cell cycle regulation and three genes associated with DNA replication were significantly altered in WT1 and p63 knockdown cells ($p < 0.005$, Table 1).

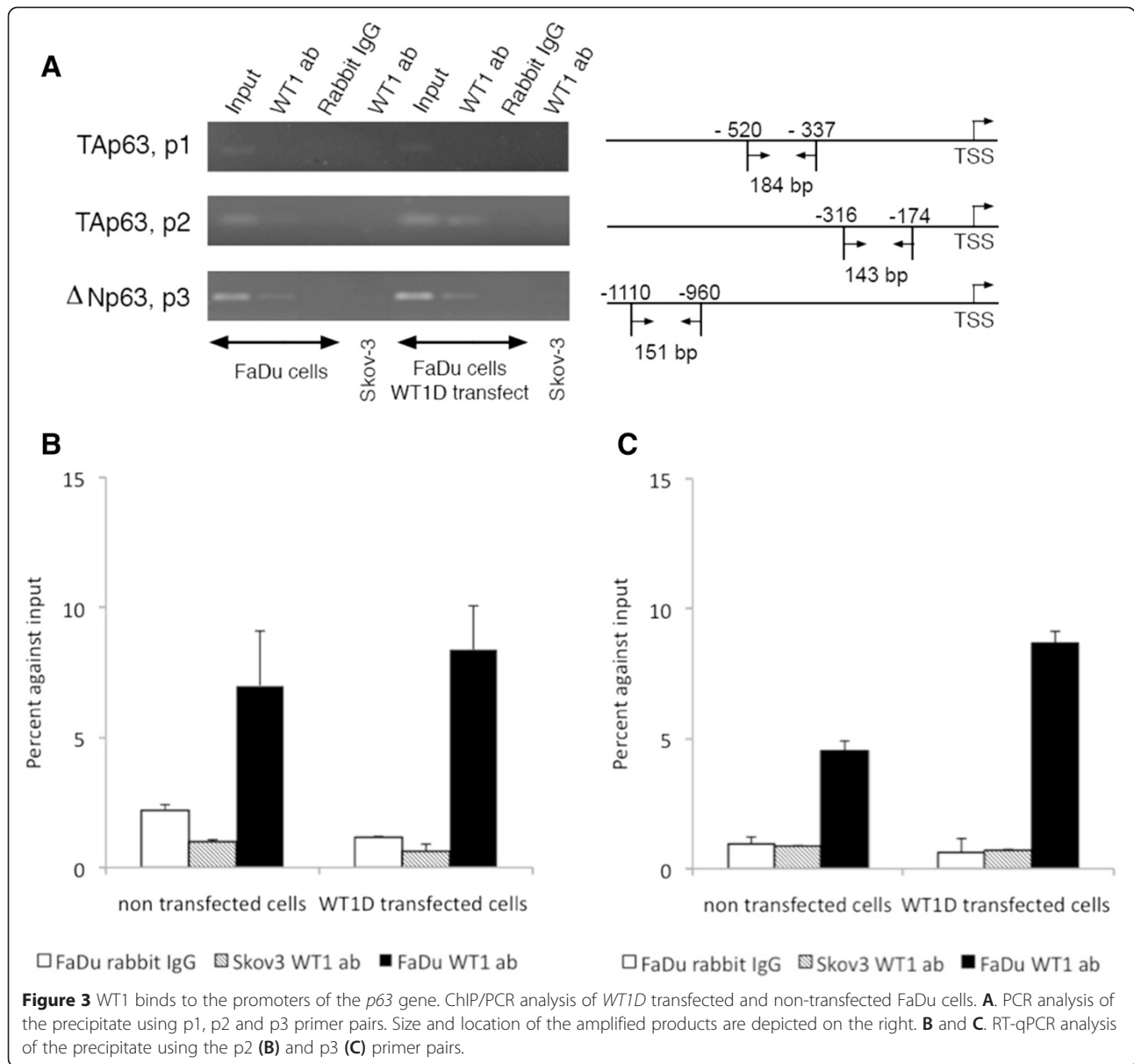
Five negative regulators of cell proliferation *IGFBP3*, *RARRES1*, *TIMP2*, *CDKN1B*, *LDOC1* and one positive regulator, *MMP7*, demonstrated increased expression. Two suppressors, *TOB2* and *SFN* and one activator, *NGFR*, showed decreased expression. *TGM2*, a positive regulator of cell cycle progression and *CI3orf15*, which has been described as both activator and suppressor of cell cycle progression, demonstrated increased expression. *Skp2*, another activator of cell cycle progression showed decreased expression. All three positive regulators of DNA replication,

MCM3, *MCM5* and *RFC3* demonstrated decreased expression. Interestingly, *IL8*, an activator of cell proliferation, demonstrated decreased expression in WT1 knockdown cells, but increased expression in p63 knockdown cells. No genes associated with apoptosis were found to be altered in the combined profiles. However, knockdown of p63 was found to induce alterations in the transcription of 24 genes involved in apoptosis.

In addition, by using Metacore GeneGo analysis, 6 known WT1 target genes and 27 known p63 downstream target genes were found to be affected in WT1 knockdown cells (Figure 4B). In p63 knockdown cells, 44 known p63 target genes were affected (Additional file 2: Figure S1). Among those p63 target genes, ten demonstrated altered expression in both WT1 knockdown and p63 knockdown cells (Table 2). Expression of four genes was significantly decreased by p63 and WT1 siRNA transfection. *SFN* is known to be repressed by p63 while *Skp2* and *CAD* can be activated by p63. In contrast, significantly increased expression of six genes was shown (Table 2). *CITED2* and *GDF2* are known to be activated by p63 whereas *PLAC8* and *IGFBP3* are repressed by p63. The effects of p63 on *Fjx1*, *INPP4B* and *TGM2* are unspecified. Taken together, these genes are known to be involved in cell cycle, cell growth, cell migration, cell proliferation, inositol phosphate metabolism and pyrimidine metabolism.

WT1 protein interacts with p53 but not p63

In order to study the protein interaction between WT1 and p53/p63, co-IP analysis was performed. As shown in Figure 5, p53 was detected in WT1 immune-complexes



but not p63, indicating protein interaction occurred between *WT1* and *p53* in FaDu cells.

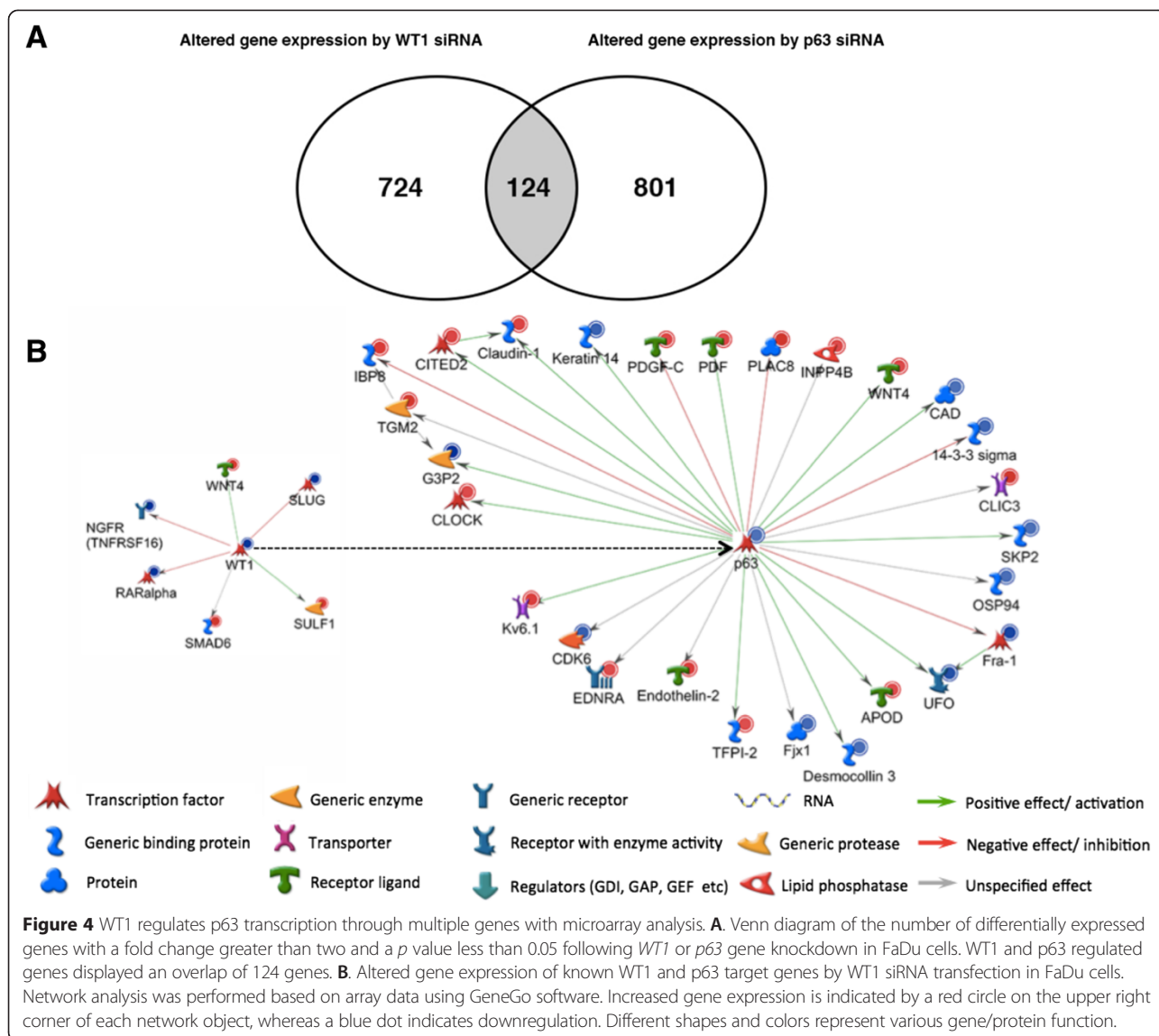
High *WT1* RNA expression in clinical samples

WT1 RNA expression levels were analyzed by real-time quantitative PCR (RT-qPCR) in 15 SCCHN tumor specimens, 7 adjacent tumor-free tissue samples and 14 normal control tissues of the tongue. Significantly higher *WT1* mRNA levels were detected in tumor specimens compared to adjacent tumor-free tissue samples (Additional file 3: Figure S2, $p < 0.001$) and normal control tongue tissues (Additional file 3: Figure S2, $p = 0.001$), indicating overexpression of *WT1* in SCCHN. No significant correlation was found between *WT1* mRNA levels and clinical features including age, sex, tumor stage, overall survival

and disease specific survival (data not shown). Using immunohistochemistry, we performed *WT1* protein staining in 90 formalin-fixed tumour samples and found that only 5 out of 90 samples showed positive staining in cytoplasm.

Discussion

In the present study we found a novel positive correlation between *WT1* and *p63* gene expression and further confirmed that *WT1* regulates *p63* expression through direct binding to the *p63* promoters. Both *WT1* and *p63* were found to promote cell proliferation in SCCHN cells. Further, *in vitro* experiments showed altered expression of 18 genes involved in cell proliferation, cell cycle regulation and DNA replication shared by silencing of *WT1* and *p63* RNA. Several known *WT1* and *p63*



target genes were affected by knockdown of *WT1*. Additionally, *WT1* mRNA levels were overexpressed in SCCHN samples.

Using *in vitro* experiments, we found decreased cell proliferation due to loss of *WT1* in FaDu cells. *WT1* isoform D was recently found to induce cell proliferation in oral squamous cell carcinoma cells, a subtype of SCCHN [28]. Furthermore, increased cell proliferation induced by *WT1* has been shown in several other types of cancer cells including non-small cell lung cancer [11] and several solid cancer cells [29]. The collected data suggest that *WT1* functions as an oncogene in these neoplasms.

Overexpression of *p63* has been found in a majority of patients with squamous cell carcinomas and SCCHN [30]. In FaDu cells, Δ Np63 has been found to be the main isoform [6,31]. One previous study has shown that knockdown of the Δ Np63 isoform, but not the TAp63

isoform inhibits cell proliferation in some SCCHN cell lines [32]. However, another study has shown that the silencing of Δ Np63 in FaDu cells does not alter the proliferation state, as judged by Ki-67 expression and FACS analysis regarding cell cycle phase DNA content [4]. In the present study decreased cell proliferation was observed in *p63* knockdown cells, showing that *p63* can promote cell proliferation in FaDu cells and overexpression of Δ Np63 isoform was detected by western blot. Our results support the expected oncogenic role of the *p63* gene in this cell line.

The FaDu cell line contains a point mutation of *p53* at codon 248 (Arg \rightarrow Leu) [26], one of the most frequent mutation sites of the gene [25]. Codon 248 is located in the DNA binding domain and mutations in this specific location has suggested generating a protein incapable of binding to target DNA, thereby losing its regulatory function on

Table 1 Significant fold changes of expression of genes involved in cell proliferation, cell cycle regulation and DNA replication by knockdown of WT1 or p63 in FaDu cells

Term	Gene name	Expected effect*	Fold change (vs control)	
			siWT1 RNA	siP63 RNA
Cell proliferation	MMP7	Activator	2.11	4.11
	NGFR	Activator	0.47	0.36
	IL8	Activator	0.46	2.22
	IGFBP3	Suppressor	2.63	2.85
	RARRES1	Suppressor	2.48	8.36
	TIMP2	Suppressor	2.12	2.07
	CDKN1B	Suppressor	2.09	2.45
	LDOC1	Suppressor	2.01	2.69
	TOB2	Suppressor	0.48	0.36
	SFN	Suppressor	0.41	0.38
Cell cycle	TGM2	Activator	4.14	4.22
	Skp2	Activator	0.49	0.46
	C13orf15	Activator/Suppressor	4.07	15.17
	SMAD6	Unspecified	3.19	3.06
	CITED2	Unspecified	2.30	3.18
DNA replication	MCM3	Activator	0.48	0.49
	MCM5	Activator	0.40	0.48
	RFC3	Activator	0.37	0.44

*Expected effect of the listed genes was based on previous studies.

transcription [33]. Failure of induction of p53-dependent apoptosis has previously been demonstrated in FaDu cells [34]. However, we observed that p53 had an inhibitory effect on cell proliferation. The same mutation in H322a, a non-small cell lung cancer cell line, showed that mutant p53^{R248L} still possesses a tumor suppressor function, as demonstrated by expansion of cell proliferation due to reduction in gene expression [35].

WT1 is known to regulate transcription of an extensive number of genes [36]. In this study we found a strong positive correlation between WT1 and p63 and confirmed that the WT1 protein binds to the p63 promoters, assessed by ChIP/PCR analysis which showed that p63 is a target gene of WT1. A direct binding of WT1 protein to the promoters of the two main p63 isoforms, TAp63 and ΔNp63 was demonstrated. However,

Table 2 Fold changes in expression of known p63 target genes in response to WT1 and p63 gene knockdown in FaDu cells

Gene name	Expected effect by p63*	Fold change (vs control)		Gene function
		siWT1	siP63	
SFN	Repressed	0.41	0.38	Cell proliferation
Skp2	Activated	0.49	0.46	Cell cycle
CAD	Activated	0.50	0.47	Pyrimidine metabolism
Fjx1	Unspecified	0.44	0.47	Cell growth
INPP4B	Unspecified	1.10	1.27	Inositol phosphate metabolism
CITED2	Activated	1.20	1.67	Cell cycle
TGM2	Unspecified	2.05	2.08	Cell cycle
PLAC8	Repressed	2.51	2.43	Cell migration
GDF15	Activated	1.18	2.66	Cell migration
IGFBP3	Repressed	2.63	2.85	Cell proliferation

*Expected effect of the listed genes was based on previous studies.

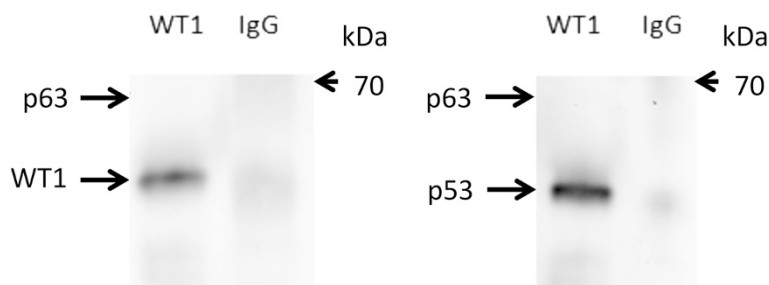


Figure 5 Protein interactions between WT1 and p53 but not p63 by co-IP. Equivalent amounts of protein lysate from FaDu cells were incubated with the anti-WT1, anti-IgG antibodies, followed by incubation with Protein G Sepharose 4 Fast Flow. Immunoprecipitated proteins were analyzed by Western blotting. Immuno-blotting was conducted using anti-WT1, p53 and p63.

the WT1 binding site (P1,-502 to -493), far from the major transcription start site in the *TAp63* promoter, was not involved. We did not find any altered *TAp63* expression in our experiment. Low efficiency may be explained by very low expression of *TAp63* in FaDu cells by western blot and only one binding site on *TAp63* promoter by WT1 protein by ChIP/PCR. As mentioned previously, $\Delta Np63$ is the only major isoform expressed and the isoform that plays a major functional role in FaDu cells.

Previous studies have presented evidence for a protein-protein interaction between WT1 and p53 in baby rat kidney [37] cells, as well as in Wilms' tumors [27]. A *p53* mutation at position homologues to human codon 248 in BRK cells did not abolish this interaction. Furthermore, WT1-induced p53 protein stabilization has been reported in Saos-2 cells [15]. In this study, we also showed that WT1 interact with p53 in FaDu cell by using Co-IP analysis and observed decreased protein levels of p53 in cells with suppressed WT1 expression at 72 hours. Results may be explained by previous findings regarding p53 protein stabilization. In contrast to previous study [16], protein interaction between WT1 and p63 was not detected in FaDu cells.

Microarray analysis showed that 18 genes involved in cell proliferation, cell cycle regulation and DNA replication were significantly altered in both WT1 and p63 knockdown cells. Five of these genes were previously described as p63 target genes. $\Delta Np63$ has been reported to directly repress the expression of the p53-target genes *IGFBP-3* [38] and *SFN (14-3-3 σ)* [39], supporting the known dominant negative effect of $\Delta Np63$ regarding p53 function [5]. *CITED2* and *Skp2* were also previously identified target genes of p63 [40,41]. *CDKN1B (p27^{kip1})* expression has been shown to be inversely correlated to $\Delta Np63$ expression, suggesting a possible direct negative regulation of $\Delta Np63$ on *CDKN1B* transcription [32]. The fold changes of 11 of these 18 genes were almost identical. An indirect regulation of p63 target genes as major mechanism for WT1 regulation of listed genes is

therefore not likely. According to immunoblot results, WT1-knockdown cells express p63 at a reduced level, still enabling transcriptional regulation as opposed to p63-knockdown cells. *MMP7*, *RARRES1*, *C13orf15* and *CITED2* are genes showing a distinct difference between WT1 and p63 knockdown cells. These genes were all shown to be repressed by both p63 and WT1, but to a greater extent by p63. Indirect regulation by WT1 might serve as regulation of those genes. *CITED2*, as mentioned previously is the only known p63 target gene of the above listed genes [40].

MMP-7 is a matrix degrading protein usually associated with tumor invasion and angiogenesis in cancer progression [42], but has also been linked to induction of proliferation [43] and apoptosis [44]. In contrast to these findings, we showed increased fold changes of *MMP7* expression in both WT1 and p63 knockdown cells. *MMP-7* has been reported to be overexpressed in SCCHN [45].

Previous studies have shown contradictory functions for the *RGC-32* gene (*C13orf15*). *RGC-32* has been reported to promote cell cycle progression and thereby cell proliferation [46]. However, tumor suppressor properties of the *RGC-32* gene have also been reported. *RGC-32* has been identified as a p53 target gene with an ability to inhibit cell proliferation by the induction of G2/M arrest [47]. *RGC-32* was found in the present study to be extensively upregulated in p63 knockdown cells. Our results suggest that *RGC-32* may act as a tumor suppressor in FaDu cells.

Interestingly, *IL8* demonstrated decreased expression when silencing WT1, but an increased fold change when knocking down p63. *IL8* is known to be a pro-inflammatory chemokine that responds to the activation of NF- κ B. *IL8* induces angiogenesis through activation of endothelial cells and has been reported to act as an autocrine growth factor inducing cell proliferation [48]. A recent study showed that $\Delta Np63$ can bind to the *IL8* promoter and alter gene expression when interacting with RelA or cRel, members of the NF- κ B family [49]. Contrary to the observations in our *in vitro* experiment, $\Delta Np63$ has previously shown to have

an activating effect on *IL8* transcription in SCCHN cells [50]. Association between *WT1* and *IL8* expression has not previously been reported. Further studies are therefore needed to investigate whether *WT1* regulates *IL8* expression directly or indirectly.

The effects of *WT1* and *p63* on cell proliferation observed in this study can be explained by their regulation of many genes involved in proliferation, cell cycle processes and DNA replication. Additionally, *WT1* was found to regulate genes involved in the *p53*, *Wnt* and *PI3K/AKT-1* signaling pathways, giving further ground for the proliferative effect of *WT1* in FaDu cells. In the present study we suggested that *WT1* could inhibit the *p53*-signaling pathway through transcriptional regulation of activators and repressors of the pathway. No alterations of apoptosis-regulating genes were found in *WT1*-depleted cells, suggesting a possible alteration of this signaling pathway through cell cycle arrest and transcriptional activation of DNA repair genes. Furthermore, in this study we could not detect any pattern of up- or downregulation of the *Wnt* or *PI3K/AKT-1* pathways. However, earlier studies have identified nine genes in the *Wnt* signaling pathway to be direct targets of *WT1* [51]. The *PI3K/AKT-1* pathway has been implicated in *WT1* signaling in lung cancer [52].

Using Metacore GeneGo software we found that expressions of ten known *p63* target genes were altered in both *WT1* and *p63* knockdown cells. These genes were involved in the cell cycle, cell growth, cell migration, cell proliferation, inositol phosphate metabolism and pyrimidine metabolism. *SFN* was previously found to be negatively regulated by $\Delta Np63$ in primary human epidermal keratinocytes (HEKs) as described above [39]. *Skp2* expression has been found positively regulated by *p63* in HEKs [41]. Using ChIP-on-chip array analysis, Huang et al. found that the $\Delta Np63$ protein could bind to the *CAD* promoter in squamous cell carcinoma cells when cells were exposed to cisplatin [53]. A previous study showed that *p63* could activate the *CITED2* promoter in keratinocytes [54]. In human keratinocytes, HaCaT, TAp63 was found to activate *GDF15* by directly binding to the promoter [55]. The proapoptotic protein IGFBP-3 has been shown to be negatively regulated by $\Delta Np63\alpha$ in the squamous epithelial cell lines HaCaT and SCC-1 [38]. However, these known *p63* target genes have not been reported correlated with *WT1*. Further studies are needed to find out whether *WT1* can directly regulate these genes.

In agreement with a study by Oji et al. [17], overexpression of *WT1* was detected in SCCHN tissue samples in our patient cohort. In a study by Mikami et al., *WT1* mRNA was found to be overexpressed in one of six cell lines from oral squamous cell carcinoma. Immunohistochemical analysis of tissue sections showed overexpression of *WT1* protein in two of 29 patients with oral squamous cell carcinoma, suggesting that *WT1* plays an important

role in the pathogenesis of some types of oral squamous cell carcinoma [56]. No correlation between *WT1* mRNA levels and clinical parameters such as age, sex, tumor stage and overall survival was observed in our limited patient cohort. The potential prognostic impact should, however, be studied in larger patient cohorts.

Conclusions

Our experimental results in FaDu cells indicate oncogenic roles for *WT1* and *p63* in SCCHN cells. We reported for the first time that *WT1* can directly regulate *p63* expression and induce an effect on several known *p63* target genes. Therefore, therapeutic approaches targeting the *WT1* and *p63* proteins might serve as alternative treatment in SCCHN. These findings may warrant further investigation regarding the effects of *WT1* and *p63* inhibitors *in vitro* and *in vivo*.

Additional files

Additional file 1: Table S1. Primers used for amplification of *p63* promoters regions.

Additional file 2: Figure S1. Altered gene expression of known *p63* target genes was found by *p63* siRNA transfection in FaDu cells. Network analysis was performed based on array data using GeneGo software. Increased gene expression is indicated by a red circle on the upper right corner of each network object, whereas a blue dot indicates downregulation. Different shapes and colors represent various gene/protein functions.

Additional file 3: Figure S2. *WT1* mRNA levels in tongue tumor tissue samples compared to adjacent tumor-free tissues or normal control tongue tissue.

Abbreviations

WT1: Wilms' tumor gene 1; SCCHN: Squamous cell carcinoma of the head and neck; siRNA: Silencing RNA; ChIP: Chromatin immunoprecipitation; Co-IP: Co-immunoprecipitation; DAVID: Database for annotation, visualization, and integrated discovery; RT-qPCR: Real-time quantitative PCR; BRK: Baby rat kidney; HEKs: Human epidermal keratinocytes.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

XL, AL conceived and designed the study. XL, SO, SW, EJ and LB performed different experiments. The data was analyzed by XL, SO, SW, EJ, LB and XG. KN contributed the materials, reagents and analysis tools. The manuscript was written by XL, SO, SW and AL. All authors were involved in revising the manuscript. All authors read and approved the final manuscript.

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