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Wilms' tumor gene 1 in different types of cancer

Xingru Li

Department of Medical Biosciences, Clinical Chemistry
Umeå University, Sweden
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To my family

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Abstract

The Wilms' tumor gene 1 (*WT1*) was first reported as a tumor suppressor gene in Wilms' tumor. However, later studies have shown the oncogenic properties of *WT1* in a variety of tumors. It was recently proposed that *WT1* was a chameleon gene, due to its dual functions in tumorigenesis. We aimed to investigate the clinical significance of *WT1* as biomarker in acute myeloid leukemia (AML) and clear cell renal cell carcinoma (ccRCC) and to elucidate the function of *WT1* as an oncogene in squamous cell carcinoma of head and neck (SCCHN).

In AML, it was suggested that *WT1* expression was an applicable marker of minimal residual disease (MRD). In adult patients with AML, we found a good correlation between *WT1* expression levels normalized to two control genes, *β-actin* and *ABL*. Outcome could be predicted by a reduction in *WT1* expression in bone marrow (≥ 1-log) detected less than 1 month after diagnosis, when *β-actin* was used as control. Also, irrespective of the control gene used, outcome could be predicted by a reduction in *WT1* expression in peripheral blood (≥ 2-log) detected between 1 and 6 months after treatment initiation.

Previous studies in RCC demonstrated that *WT1* acted as a tumor suppressor. Thus, we tested whether single nucleotide polymorphisms (SNPs) or mutations in *WT1* might be associated with *WT1* expression and clinical outcome in patients with ccRCC. We performed sequencing analysis on 10 exons of the *WT1* gene in a total of 182 patient samples, and we identified six different SNPs in the *WT1* gene. We found that at least one or two copies of the minor allele were present in 61% of ccRCC tumor samples. However, no correlation was observed between *WT1* SNP genotypes and RNA expression levels. Moreover, none of the previously reported *WT1* mutations were found in ccRCC. Nevertheless, we found that a favorable outcome was associated the homozygous minor allele for *WT1* SNP. We then further investigated whether *WT1* methylation was related to *WT1* expression and its clinical significance. Methylation array and pyrosequencing analyses showed that the *WT1* promoter region CpG site, cg22975913, was the most frequently hypermethylated CpG site. We found a trend that showed nearly significant correlation between *WT1* mRNA levels and hypermethylation in the 5'-untranslated region. Hypermethylation in the *WT1* CpG site, cg22975913, was found to be associated with patient age and a worse prognosis.

One previous study reported that *WT1* was overexpressed in SCCHN. That finding suggested that *WT1* might play a role in oncogenesis. We found that both WT1 and p63 could promote cell proliferation. A positive correlation between WT1 and p63 expression was observed, and we identified *p63* as a WT1 target gene. Furthermore, several known WT1 and p63 target genes were affected by knocking down WT1. Also, co-immunoprecipitation analyses demonstrated a protein interaction between WT1 and p53.

In summary, *WT1* gene expression can provide useful information for MRD detection during treatment of patients with AML. In RCC, our results suggested that the prognostic impact of *WT1* SNPs was limited to the subgroup of patients that were homozygous for the minor allele, and that *WT1* promoter hypermethylation could be used as a prognostic biomarker. In SCCHN, WT1 and p63 acted as oncogenes by affecting multiple genes involved in cancer cell growth.

Original Articles

This thesis is based on the following papers and manuscripts:

I. Andersson C, **Li** X, Lorenz F, Golovleva I, Wahlin A, Li A. Reduction in WT1 gene expression during early treatment predicts the outcome in patients with acute myeloid leukemia. *Diagn Mol Pathol*. 2012 Dec; 21(4):225-33.

II. **Li X**, Wang S, Sitaram RT, Andersson C, Ljungberg B, Li A. Single nucleotide polymorphisms in the Wilms' tumour gene 1 in clear cell renal cell carcinoma. *PLoS One*. **8(3):**e58396. doi: 10.1371/journal.pone.0058396. 2013

III. **Li X**, Evelönn E A, Wang S, Sitaram RT, Landfors M, Ottosson S, Andersson C, Nilsson S, Ljungberg B, Li A. Prognostic significance of hypermethylation in the promoter region of the Wilms' tumour gene 1 in clear cell renal cell carcinoma. *Manuscript*.

IV. **Li X**, Ottosson S, Wang S, Jernberg E, Boldrup L, Gu X, Nylander K, LiA. Wilms' tumor gene 1 regulates *p63* and promotes cell proliferation in head and neck squamous cell carcinoma. *BMC Cancer*. 2015 May 1; 15(1):342.

Abbreviations

5’-UTR	5'-untranslated region
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
BM	bone marrow
BRK	baby rat kidney
ccRCC	clear cell renal cell carcinoma
CG	control gene
CR	complete remission
DDS	Denys–Drash syndrome
DSS	disease specific survival
FFR	freedom from relapse
HaCaT	human keratinocyte cell line
KTS	lysine, threonine and serine
MRD	minimal residual disease
MSP	methylation-specific PCR
OS	overall survival
PB	peripheral blood
PCR	polymerase chain reaction
RCC	renal cell carcinoma
RQ-PCR	real-time quantitative-PCR
SCCHN	squamous cell carcinoma of the head and neck
SNP	single nucleotide polymorphism
T-ALL	T-acute lymphoblastic leukaemia
UTSS	upstream of the transcription start site
WT1	Wilms’ tumor gene 1

Introduction

WT1 (Wilms' tumor gene 1)

Wilms' tumor gene 1 (*WT1*) was first reported as a tumor suppressor gene in Wilms' tumor, a childhood kidney neoplasm [1]. Later findings demonstrated that *WT1* had oncogenic properties in other malignancies, including breast [2], lung [3], ovarian [4], and brain [5] cancers. *WT1* is an important regulatory molecule involved in cell growth and development. It is expressed in a tissue-specific manner. In the developing embryo, *WT1* expression is found primarily in the urogenital system. In adult tissues, *WT1* expression is found in the urogenital system, the central nervous system, and in tissues involved in hematopoiesis, including bone marrow and lymph nodes [6].

Structure of WT1

The *WT1* gene is located in the chromosome locus 11p13. It encodes a 3 kb mRNA and consists of 10 exons [7]. Two alternative splicing events produce four different WT1 protein isoforms that vary in size between 52 and 54 kDa. Alternative splicing at site I inserts 17 amino acids in exon 5; alternative splicing at site II inserts three amino acids (lysine, threonine, and serine [KTS]) in exon 9. The four different protein isoforms are designated: A (–/–), B (+/–), C (–/+), and D (+/+), where the signs indicate the presence or absence of the two amino acid inserts [8]. The C-terminal domain of WT1 is composed of four Krüpple-like, cysteine$_2$-histidine$_2$ zinc fingers, which are involved in RNA and protein interactions; these interactions permit binding to target DNA sequences [9]. The KTS insert interrupts the spacing between zinc-fingers three and four, which alters the binding specificity of the protein to target DNA (Figure 1). The N-terminal domain of WT1, comprised of proline–glutamine-rich sequences, is also involved in RNA and protein interactions.

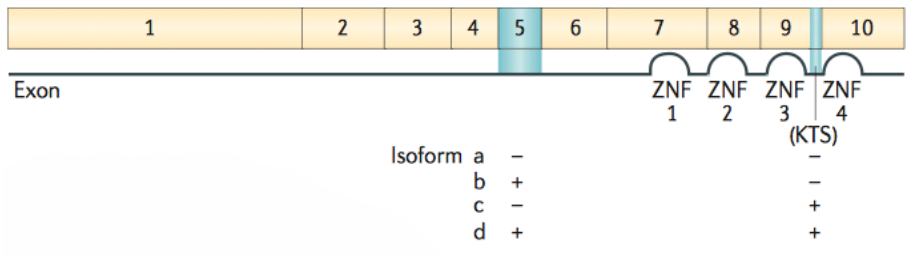

Figure 1. *WT1* gene and protein structure. Blue shading indicates alternatively spliced domains. Four isoforms result from exon 5 and KTS alternative splicing. Adapted from Huff, 2011 Nature Reviews Cancer [10].

WT1, the transcription factor

WT1 was identified as a transcription factor by the presence of zinc fingers in the C-terminal domain (Figure 2). An extensive number of genes were found to be regulated by *WT1*, including genes that code for growth factors (*IGF-2*, erythropoietin, *PDGF-A*), growth factor receptors (*IGF-1-R*, *EGF-R*), cell cycle control proteins (*c-myc*, *p21*, *cyclin E*), apoptosis-regulating proteins (*bcl-2*), development-related proteins (*Dax-1*, *Sry*), and others (*SMAD3*, *hTERT*; Table 1). WT1 exerts activation or repression of these genes, depending on promoter context and cellular state [11]. WT1 was found to have dual effects on *c-myc* and *bcl-2* expression; WT1 could either activate or repress their expression, depending on the cell type [12, 13]. WT1 activated the transcription of *c-myc* in breast cancer, but it inhibited *c-myc* transcription in renal cell carcinoma (RCC) [13, 14].

WT1 and its interacting partners

Several proteins have been found to associate with and regulate WT1 (Table 2). Protein-protein interactions have demonstrated the ability to modify the functions of WT1 in regulating gene transcription [15]. An interaction between WT1 and tumor suppressor protein, p53, was involved in p53 protein stabilization in baby rat kidney (BRK) cells and in an osteosarcoma cell line, Saos-2 [16] (Table 2). The protein product of the *p53* gene is one of several proteins that bind to the WT1 protein and alter its transcription regulatory properties. The interaction between WT1 and p53 was shown to modulate the ability of both proteins to regulate transcription

Table 1. Target genes transcriptionally regulated by WT1

Target gene	Expected effect	References
Growth factors		
Erythropoietin	Activation	[17]
Amphiregulin	Activation	[18]
Insulin-like growth factor II	Activation	[19]
Connective tissue growth factor	Repression	[20]
Platelet-derived growth factor A	Repression	[21]
Colony-stimulating factor-1	Repression	[22]
Transforming growth factor-beta	Repression	[23]
Growth factor receptors		
Erythropoietin receptor	Activation	[24]
Insulin receptor	Repression	[25]
Insulin-like growth factor 1 receptor	Repression	[26]
Androgen receptor	Repression	[27]
Estrogen receptor-A	Repression	[28]
Epidermal growth factor receptor	Repression	[29]
Cell cycle regulators		
c-myc	Activation	[14, 30]
p21	Activation	[31]
Retinoblastoma suppressor associated protein 46	Activation	[32]
cyclin E	Repression	[33]
ornithine decarboxylase	Repression	[34]
Apoptosis regulators		
bcl-2	Activation	[12]
A1/BFL1	Activation	[35]
Bak	Activation	[36]
JunB	Repression	[37]
Development-related genes		
Dax-1	Activation	[38]
SRY	Activation	[39]
anti-Müllerian hormone receptor 2	Activation	[40]
Sprouty 1	Activation	[41]
nestin	Activation	[42]
Pou4f2	Activation	[43]
TauT	Activation	[44]
WT1-induced inhibitor of Dishevelled	Activation	[45]
TrkB neurotrophin receptor	Activation	[46]
Others		
vitamin D receptor	Activation	[47]
E cadherin	Activation	[48]
Syndecan-1	Activation	[49]
human telomerase reverse transcriptase	Repression	[13, 50]
SMAD3	Activation	[13]

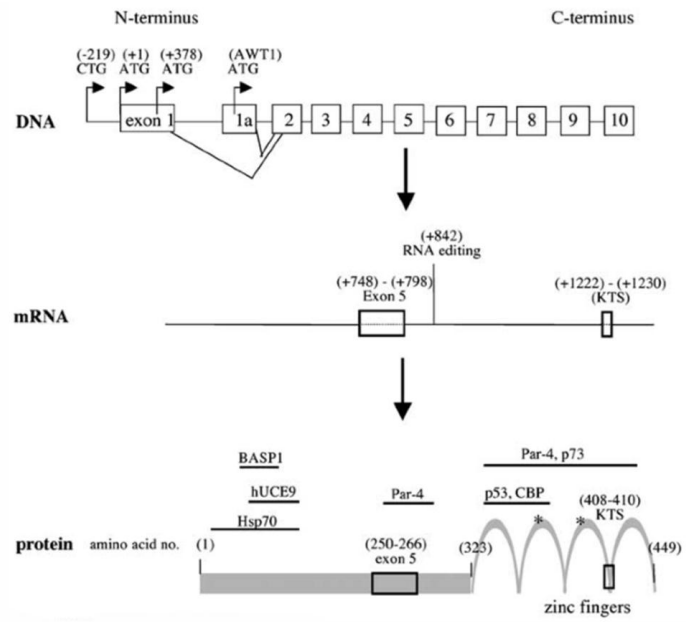

Figure 2. Schematic diagram of WT1 DNA (exons only), mRNA, and protein structures. [51] *WT1* can be transcribed from four initiation start sites. The most recently reported WT1 isoform, AWT1, is truncated, due to transcription from an internal ATG, located within the intron that lies between exon 1 and exon 2. Several post-transcriptional modifications occur in WT1. (*Middle*) At the mRNA level, *WT1* RNA is edited at nucleotide position 843. Also, it is subject to RNA splicing, which can remove 51 nucleotides of exon 5 and/or nine nucleotides between exons 9 and 10. These splicing events yield the four major isoforms of WT1. (*Bottom*) The WT1 protein has several functional domains. The N-terminal domain is proline–glutamine-rich, and it contains transcriptional repression and activation domains. In addition, WT1 contains self-association and RNA recognition motifs. The C-terminal domain contains four C2H2 Krupple-like zinc-fingers. In addition to binding DNA and some proteins, these zinc fingers can regulate RNA targets and mediate nuclear localization. The numbers shown in parentheses above the DNA and RNA schematics represent nucleotide sequences relative to the first ATG codon, and the numbers above the protein schematic represent the amino-acid sequence. All numbers correspond to the human WT1 (+/+) isoform. Lines shown above the WT1 protein schematic indicate regions reported to be involved in WT1 interactions with selected regulatory molecules (labeled). Asterisks (*) indicate reported phosphorylation sites at serine 365 and serine 393, located in zinc-fingers 2 and 3, respectively. The schematics are not drawn to scale. Adapted from Yang et al., 2007 Leukemia [11].

of their respective target genes. This suggested that associations between WT1 and members of the p53 family may be an important determinant of their functions in cell growth and differentiation [134]. More recently, p63 was identified as a protein partner of WT1, a member of the p53 family of transcription factors [52]. *p63* exhibits nucleotide homology with *p53* in DNA binding, oligomerization, and transactivating domains.

WT1 function

The tumor suppressor

WT1 was initially discovered as a tumor suppressor gene in Wilms' tumor [53]. It was found that, in the majority of cases, Wilms' tumors expressed wild-type *WT1*, sometimes to high levels. In fact, in the sporadic form of the disease, only 10% had a *WT1* mutation. Those findings suggested that *WT1* mutations are important only in a small fraction of cases. However, three Wilms' tumor-related syndromes were associated with germline mutations or deletions in *WT1*: WAGR syndrome (Wilms' tumor, ANIRIDIA, genitourinary abnormalities, and mental retardation) [7, 54, 55], Denys–Drash syndrome (DDS) [56], and Frasier syndrome [57, 58]. The function of *WT1* as a tumor suppressor has been studied with different models *in vitro* and *in vivo*. WT1 expression conferred the ability to suppress cell proliferation in the Wilms' tumor cell line, G401, and to induce programmed cell death in osteosarcoma cell lines [29, 59, 60]. WT1 has also been found to induce apoptosis in the Saos-2 cell line and B16F10 melanoma cells [36, 61]. In mice, WT1 could induce growth suppression, reduce tumor formation, and suppress tumorigenicity [62-64]. In addition, overexpression of WT1 was found to induce cell cycle arrest and apoptotic cell death in M1 leukemia cells (Figure 3) [63].

An oncogene

Overexpression of *WT1* has been demonstrated in carcinomas from a variety of origins, including lung cancer [3, 65], breast cancer [2], colon cancer [66], pancreatic cancer [67], ovarian cancer [68, 69], brain tumors [5], pediatric sarcomas [70], and other tumors. It has been suggested that *WT1* might play a role in oncogenesis. Results from functional studies support this hypothesis. Algar et al. demonstrated that *WT1* inhibition with an antisense oligonucleotide (RNAi) approach reduced cell proliferation, migration, and endothelial tube formation in endothelial cells [71]. In breast cancer cells, ablation of the WT1 protein led to apoptosis and cell cycle arrest at G1 [72]. The 17AA(+) WT1 isoform showed anti-apoptotic potential in leukemia cells and other solid tumor cells, including lung tumors, gastric, ovarian cancers, fibrosarcoma, and glioblastoma [73, 74]. The oncogenic role of *WT1* has been extensively studied in leukemia. Overexpression of *WT1* has been reported in acute myeloid leukemia (AML), chronic myeloid leukemia,

Table 2. Selected protein partners of WT1

Interacting protein	WT1 interacting domain	Function/consequences
BASP1	71-101 aa	Transcriptional co-suppressor
HtrA2	71-101 aa	Serine protease/apoptosis dependent cleavage of WT1
hUBC9	85-179 aa	SUMO-1 E2-conjugating enzyme/sumoylation of WT1
Hsp70	1-180 aa	Decreased proliferation
PAX2	1-466 aa	Joint co-expression in renal development/unknown
STAT3	1-281 aa	Promotes cell proliferation
SF1	N-terminus	Co-activates MIS expression with WT1 (-KTS) /regulation of sexual differentiation
WT1	1-180 aa	WT1 protein self-association/dominant negative effect with WT1 mutations
Par-4	+17 AA Zn-fingers	Involved in apoptosis/co-activator with WT1 (+17AA) and lowered transcriptional activity (Zn-finger interaction)
BMZF2	Zn-fingers	TF regulating expression in fetal tissues/represses WT1 activation
CBP	Zn-fingers	Transcriptional cofactor/enhances WT1 transcriptional activity
Ciao 1	Zn-fingers	WD40 protein /inhibition of WT1 transactivation, no influence on WT1 repression
E1B55K	Zn-fingers	Adenovirus protein/WT1-induced cell death is prohibited
HCMV-1E2	Zn-fingers	Human cytomegalovirus protein/WT1 inhibits 1E2 transactivation ability
p53	Zn-fingers	Tumor suppressor/p53 stabilization and inhibited apoptosis, inhibited WT1 activation
p73	Zn-fingers	Cell cycle regulator, involved in apoptosis/inhibited transcriptional activity for p73 and WT1 (no DNA-binding)
SRY	Zn-fingers	Regulator of sex determination/synergistic transcriptional activation
U2AF65	Zn-fingers	Splicing factor/WT1 (+KTS) a component in pre-mRNA splicing
WTAP	Zn-fingers	Undetermined/WT1-interacting protein with unknown significance

Abbreviations and references: BASP1: Brain acid soluble protein 1 [75], HtrA2: High temperature requirement protein A2 [76], hUBC9: human Ubiquitin-conjugating enzyme 9 [77, 78], Hsp 70: Heat shock protein 70 [79], PAX2: Paired box 2 [80], STAT3: Signal transducer and activator of transcription 3 [81], SF1: Steroidogenic factor 1 [82], Par-4: Prostate apoptosis response factor 4 [83], BMZF2: Bone marrow zinc finger 2 [84], CBP: CREB binding protein [85], Ciao 1: Cytosolic iron-sulfur protein assembly 1 homolog [15], E1B55K [86], HCMV-1E2 [87], p53 [88], p73 [52], SRY: Sex-determining region of the Y chromosome [89], U2AF65 [90], WTAP: WT1 associating protein [91].

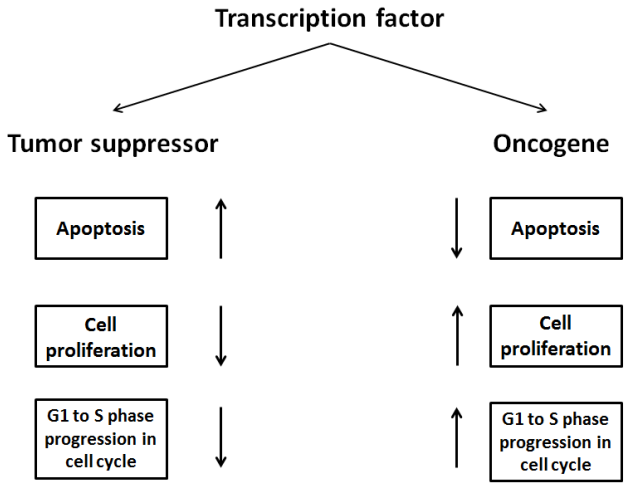

Figure 3. *WT1* gene functions in cancer. WT1 expression can suppress or activate cancer, depending on the cell type and environment.

acute lymphoblastic leukemia (ALL), and myelodysplastic syndrome [92-94]. In myeloid leukemia cell lines, K562 and MM6, the knockdown of WT1 gene expression induced cell proliferation inhibition and apoptosis (Figure 3) [74, 95].

Mutations and single nucleotide polymorphisms (SNPs) in *WT1*

WT1 mutations

Sequencing analyses demonstrated that *WT1* mutations occurred in only 10% of sporadic Wilms' tumors [96]. However, mutations in the *WT1* gene are frequently found in anomalous urogenital syndromes, such as DDS [97, 98] and Frasier syndrome [57, 99]. Several studies in both adult and pediatric patients have shown *WT1* mutations in approximately 10% of T-acute lymphoblastic leukemia (T-ALL) [100, 101] and AML cases [102-107]. Abnormalities found in Wilms' tumor include intragenic mutations and large deletions [108]. In DDS, the majority of WT1 alterations are point mutations in zinc finger 2 or 3 [109, 110]. Frasier syndrome was associated with point mutations at the second splice donor site in intron 9 [58]. AML- associated *WT1* mutations were predominantly insertions or deletions in a mutation hotspot on exon 7 or exon 9, and less commonly, missense mutations in exon 9 (Figure 4). In AML, *WT1* heterozygous and homozygous mutations have

been reported at different frequencies [103, 105]. In patients with T-ALL, mainly heterozygous frameshift mutations were observed [100]. It remains unclear how a *WT1* mutation might contribute to leukemia development. In RCC, lower *WT1* expression was found in tumor samples compared to the tumor-free kidney cortex; that finding suggested that *WT1* may play a tumor suppressor role. Genetic abnormalities may be related to decreased *WT1* expression; however, this possibility remains to be investigated.

SNPs in WT1

Unlike DNA mutations, synonymous SNPs encode a substitution in the DNA sequence without altering the resultant protein [111]. These substitutions were assumed to be unimportant, until recently. Recent studies have shown that synonymous SNPs may alter gene functions and phenotype through mechanisms such as alterations in mRNA splicing, stability, expression and in protein folding [112]. Furthermore, it has been reported that synonymous SNPs are associated with more than 40 diseases [113]. In the *WT1* gene, many studies have focused on the SNP, rs16754, located in exon 7. This exon has two alleles that can harbor the nucleotide adenine (A) or guanine (G); the result is a homozygous ($WT1^{AA}$ or $WT1^{GG}$) or heterozygous ($WT1^{AG}$) genotype. The minor allele frequency of *WT1* SNP rs16754 ($WT1^{AG}$ or $WT1^{GG}$) has been reported in approximately 27% of patients with AML [106, 114-116].

Methylation of *WT1*

DNA methylation

DNA methylation is important in embryonic development; it is functionally involved in genomic imprinting and X-chromosome inactivation [117-119]. Approximately 70% of gene promoters harbor short regions of high CpG-density, known as CpG islands [120]. In the human genome, DNA methylation mainly occurs on cytosine residues of CpG dinucleotides in promoter regions, and this event is often associated with gene inactivation [121]. Studies have shown that hypermethylation of CpG islands in the promoter regions of genes *p16*/*CDKN2* and $p15^{INK4B}$ was correlated with silenced gene expression in bladder tumors and hematological malignances [122, 123]. In contrast, methylation of CpG sites within the *PAX6* gene body did not block gene expression in bladder and colon tumors [124].

WT1 methylation in different types of cancer

Methylation of the *WT1* gene has been studied in different types of cancer. In colorectal cancer tissues, the *WT1* promoter region was significantly hypermethylated compared to normal colonic mucosa [125]. However, the significance of this hypermethylation was not clear, because no difference in *WT1* expression was found between normal and malignant samples. In rat mesothelioma and RCC cell lines, methylation was reported in *WT1* intron 1 [126]. In breast cancer, one previous study showed hypermethylation in the *WT1* promoter region in 25% of breast tumor specimens and in several breast cancer cell lines [127]. In breast cancer cells, *WT1* expression was restored after treatment with a demethylating agent, indicating that methylation of the *WT1* promoter was associated with gene silencing. Another study showed hypermethylation in the *WT1* promoter in 32% primary breast tumors, which showed no detectable *WT1* expression [2]. In Wilms' tumor, methylation was not detected in the *WT1* promoter region, but it was detected in one CpG site in the enhancer region [128]. Moreover, the *WT1* promoter was shown to be significantly methylated in ovarian clear cell adenocarcinoma compared to serous adenocarcinoma [129]. In both those tumor samples and in ovarian cancer cell lines, a correlation was found between *WT1* promoter methylation and *WT1* gene expression. In non-small cell lung cancer, hypermethylation was detected in the 5'-untranslated region (5'-UTR) of the *WT1* gene; moreover, methylation was associated with tumor histotype and smoking [130]. Recently, Guillaumet-Adkins et al. reported that hypermethylation of the *AWT1* promoter (an alternative *WT1* transcript) was associated with gene silencing in AML and myeloid-derived hematological cancer cell lines [131]. Furthermore, hypermethylation of *AWT1* was a differentiating factor between patients that developed relapses and those that achieved complete remission (CR). However, *WT1* methylation has not been studied in RCC.

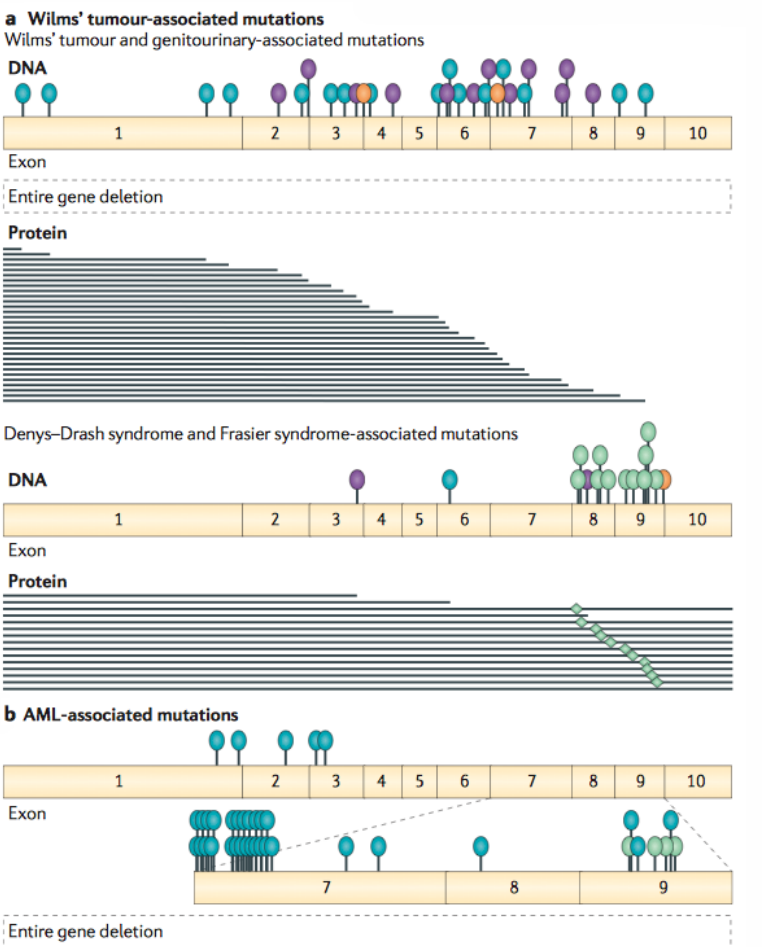

Figure 4. *WT1* mutations observed in Wilms' tumor and acute myeloid leukemia (AML) a| Germline mutations observed in patients with Wilms' tumor-associated phenotypes. Similar mutations are observed somatically in tumors. b| Somatic mutations observed in AML. Locations of gene mutations are indicated with color-coded ovals; blue: insertions, deletions, and frameshift mutations; purple: nonsense mutations; green: missense mutations; and orange: splice site mutations. The predicted sizes of mutant proteins are shown for deletion mutations. Asterisk indicates a reduced KTS+/KTS– isoform ratio as a result of IVS9 splice site mutations. ZNF, zinc finger domain. Adapted from Huff, 2011 Nature Reviews Cancer [10].

Different methods for detecting DNA methylation

Several methods have been used to detect DNA methylation [132, 133].

Genome-scale DNA methylation analysis methods

Genome-scale DNA methylation analyses include the Illumina HumanMethylation450 array assay and whole-genome bisulfite sequencing; both these methods are used extensively. The HumanMethylation450 array has the advantages of high sensitivity, high accuracy, and relatively low cost [134]. The methylation array analysis introduced the delta beta value to identify differential gene methylation between samples and controls. A cut-off level of 0.2 is commonly used [135, 136].

Locus-specific DNA methylation detection methods

Methylation-specific PCR (MSP) is commonly applied for qualitative analysis of locus-specific DNA methylation. This method has the advantage of high sensitivity [137, 138]. However, MSP can give false-positive results, due to polymerase chain reaction (PCR) amplifications. Alternatively, semi-quantitative analysis can be performed with bisulfite DNA sequencing, but this method is not often used, due to labor and cost intensities [139]. In contrast, pyrosequencing offers both quantitative DNA methylation data and single nucleotide resolution for specific genes, and it has the advantages of high sensitivity and reproducibility [140]. Therefore, pyrosequencing has recently been proposed as the gold standard for the detection of de novo DNA methylation [133].

Clinical relevance of the *WT1* gene

WT1 in acute leukemia

AML is a heterogeneous disease with a wide spectrum of phenotypes. High *WT1* mRNA expression is associated with fewer remissions, poor disease-free survival, and poor overall survival (OS) [141-144]. However, some studies have found no prognostic relevance for the presence or absence of *WT1* expression or the level of *WT1* expression (low versus high) [145, 146]. Also, in one AML study, patients with higher levels of *WT1* were found to have better OS [147]. *WT1* is mutated in 10% of both adult and pediatric AML cases [102-107]. Studies in patients with AML have shown that *WT1* mutations were significantly associated with worse relapse-free survival and OS. In T-ALL, *WT1* mutations were detected in 13.2% of pediatric and 11.7% of adult cases; however, no prognostic effect could be found for *WT1* mutations [100]. Recently, clinical interest has been raised regarding the prognostic impact of SNP rs16754 in *WT1* exon 7 for patients with leukemia. In a German study, among cytogenetically normal patients with AML, those

that had rs16754 ($WT1^{AG}$) and rs16754 ($WT1^{AA}$) genotypes were found to have a better outcome compared to patients with the rs16754 ($WT1^{GG}$) genotype [106]. However, in a large Cancer and Leukemia Group study, patients with AML that had the rs16754 ($WT1^{GG}$) genotype had a more favorable outcome among a subset of patients with FLT3-ITD [116]. Nevertheless, in a Korean cohort, the different genotypes of rs16754 did not have any significant impact on clinical outcome in AML [148].

Cytogenetic alterations, molecular genetic defects, and minimal residual disease (MRD) are patient-related prognostic factors in AML. Current treatment protocols are based on these prognostic factors, which contribute to individualized therapy and risk-adapted intensification [149]. The importance of MRD detection has grown with regard to risk of relapse and early identification of treatment responses in leukemia [150]. *WT1* has been identified as a molecular marker for MRD with the use of highly sensitive, real-time quantitative-PCR (RQ-PCR) detection [102, 141]. However, in this assay, WT1 expression levels are thought to be influenced by the region of *WT1* gene amplified and by the different control genes (CGs) used for signal normalization [151].

WT1 in RCC

RCC accounts for 3% of adult malignancies, and it is associated with a high mortality rate. The three main subtypes of RCC are clear cell (ccRCC), papillary, and chromophobe RCC [152]; however, ccRCC represents 80–90% of all RCCs [153]. RCC samples had lower *WT1* expression than tumor-free kidney cortex samples in several studies [13, 154]. However, high *WT1* expression was demonstrated in some RCC samples and in several RCC derived cell lines. These findings argued against *WT1* acting as a tumor suppressor in this tumor type [155]. An *in vitro* study in the RCC TK-10 cell line demonstrated that down-regulated *WT1* gene expression was negatively correlated to *hTERT* and *c-myc* expression [13]. These results suggested that *WT1* may play the role of a tumor suppressor gene. However, no studies have investigated *WT1* inactivation or elucidated its role in the pathogenesis of RCC. *WT1* gene inactivation may result from genetic mutations, including deletions, insertions, missense or nonsense mutations, and splice junction alterations or epigenetic abnormalities, such as hypermethylation.

WT1 in squamous cell carcinoma of the head and neck (SCCHN)

SCCHN is the sixth most common cancer and the most common tumor type in the head and neck region. The 5-year survival is approximately 50%, and

the survival rate has increased only marginally during the last few decades. The molecular pathogenesis of SCCHN remains incompletely understood, which has complicated the development of new therapeutic approaches [156]. Overexpression of *WT1* was previously demonstrated in 75% of patients with SCCHN [157]. High *WT1* expression was significantly related to poor histological SCCHN tumor differentiation and high SCCHN tumor stages. Those findings suggested that wild-type *WT1* might play an important role in the tumorigenesis of SCCHN. However, that result has not been confirmed in any other study, and the function of WT1 has not been investigated in SCCHN.

p53/p63 in SCCHN

Mutations in the *p53* gene have been reported in 1/3 to 2/3 of SCCHN cases [158]. Moreover, overexpression of the *p53* gene is frequent in SCCHN; however, controversy remains regarding the prognostic significance of *p53* overexpression. The *p53*-related transcription factor, *p63*, is reported to be overexpressed in the majority of primary SCCHN tumors [159, 160]. The *p63* gene has high sequence homology with *p53* in the DNA binding, oligomerization, and transactivating domains. The two main isoforms of p63 proteins are transcribed from two different promoters. TAp63, transcribed from the first promoter, has tumor-suppressive properties; it can bind to p53 target genes and activate their transcription. ΔNp63 is transcribed from the second promoter; it lacks the transactivating domain. ΔNp63 acts in a dominant negative fashion; it has the ability to overcome the cell-cycle arrest and apoptosis functions normally driven by p53 [161]. In contrast to the ubiquitously expressed p53, p63 displays a tissue-specific expression profile. Its expression is confined to the basal compartment of epithelial cells from skin, vagina, cervix, prostate, breast, and esophagus, where it regulates proliferation and differentiation. As mentioned above, WT1 was previously found to interact with p53 and p63 at the protein level in BRK cells and in osteosarcoma Saos-2 cells [16, 52]. However, these interactions have not been studied in any other cell types to date.

Aims of the thesis

The aim of this thesis was to investigate the clinical significance of *WT1* as a biomarker in AML and ccRCC, and to study the function of *WT1* as an oncogene in SCCHN.

Specific Aims

Paper I

- To detect *WT1* mRNA level in peripheral blood and bone marrow samples derived from patients with acute leukemia during treatment.

- To determine whether *WT1* can be used as molecular marker to predict patient outcome.

Paper II

- To study the correlation between mutation and expression levels of the *WT1* gene.

- To test the clinical relevance of *WT1* mutation in ccRCC.

Paper III

- To determine whether *WT1* methylation is related to mRNA expression levels.

- To investigate whether *WT1* can be used as a biomarker to predict patient outcome in ccRCC.

Paper IV

- To study the oncogenic function of WT1 in the regulation of *p63*/*p53*, with SCCHN as a working model.

Materials and methods

Tumor samples and cell culture (paper I - IV)
In paper I, the study included 43 adult patients (median age 61 y, range 23 to 85 y) diagnosed with AML between 1996 and 2002. These patients were treated at the Department of Hematology, Umeå University Hospital, according to standard protocols. Bone marrow (BM) and peripheral blood (PB) samples were obtained at diagnosis and during treatment. Expression levels of *WT1* mRNA were quantified at diagnosis and during follow-up in a total of 43 BM samples and in 14 PB samples.

In papers II and III, tumor samples were collected from patients diagnosed with ccRCC. These patients were treated at Umeå University Hospital, Umeå, Sweden, based on guidelines from the European Association of Urology [162]. The histological grading of specimens was performed according to the Fuhrman system. Tumor stages were classified according to the 2002 TNM classification [163]. Follow-up medical records of the patients were used for survival analysis. In paper II, the study included 182 adult patients diagnosed between 1985 and 2007. A total of 260 tissue specimens, including 182 ccRCC tumor samples and 78 corresponding tumor-free renal cortical tissue samples were sequenced over the *WT1* exons. In paper III, the study included 117 adult patients diagnosed between 2001 and 2009. Tumor specimens from 117 patients and 10 corresponding tumor-free renal cortical tissue samples were analyzed with a Genome-wide DNA methylation array. Pyrosequencing analysis was performed with paired samples of tumors and corresponding tumor-free renal cortical tissues from 33 patients with ccRCC (included in the methylation array analysis).

In paper IV, the study included tumor biopsies from 15 patients with SCCHN. Also, adjacent tumor-free tissue was available from 7 of the patients. Additionally, punch biopsies were analyzed from 14 healthy non-smoking volunteers.

All samples were collected after obtaining informed written consent. All studies were approved by the Human Ethics Committee of the Medical Faculty, Umeå University, Sweden.

In paper IV, the study analyzed the FaDu cell line (ATCC HTB-43), derived from hypopharyngeal squamous cell carcinoma.

RNA extraction and cDNA preparation (papers I - IV)
Total RNA was extracted with the TRIzol method (Invitrogen AB, Stockholm, Sweden). After extraction and isolation, the RNA concentration was determined by measuring the optical density at 260 nm, and the RNA samples were stored at -80 ºC until use. cDNA was synthesized by reverse transcription with the Superscript II Reverse Transcriptase kit, according to the manufacturer's protocol (Invitrogen).

***WT1* mRNA Expression with RQ-PCR (papers I - IV)**
Quantitative analysis of *WT1* mRNA expression was performed with TaqMan technology in the 7900 HT Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Amplification conditions, primers, and probes for the *WT1* gene and the two CGs (the *β-actin* gene and the *ABL1* gene) were described in paper I. *WT1* transcription values were normalized against the expression of *β-actin* (papers I - IV) and *ABL1* (paper I) to adjust for variations in RNA isolation and cDNA synthesis. Relative expression levels were calculated as the mean of triplicate determinations of the *WT1* gene copy number divided by the mean of duplicate determinations of the copy numbers of the CGs. As an internal experimental control, RNA from K562 cells was reverse-transcribed to produce cDNA for the RQ-PCR assay.

Genomic DNA Preparation (papers II and III)
According to the manufacturer's instructions, genomic DNA was extracted from frozen tissue specimens with the MagAttract DNA Mini M48 Kit and a Qiagen BioRobot M48 (QIAGEN, Hilden, Germany). After extraction and isolation, the DNA was stored at -80 ºC until use.

Sequencing Analysis of the *WT1* Genes (paper II)
With intron-exon flanking primer pairs, the PCR technique was applied to amplify the entire coding region of the *WT1* gene, which comprised 10 exons. The 12 primer pairs were described previously [53]. Amplification conditions were described in paper II. Sequence reactions were analyzed on an Applied Biosystems 3730×l DNA Analyzer (Applied Biosystems). Nucleotide sequences were aligned with Sequencher software, v4.7 (Gene Codes Corporation, Ann Arbor, MI, USA). The derived *WT1* gene sequences were identified by comparisons with the corresponding reference genes in GenBank (EMBL) (http://www.ncbi.nlm.nih.gov/genbank/), with the

search tools, BLAST (http://blast.ncbi.nlm.nih.gov/) and dbSNP (http://www.ncbi.nlm.nih.gov/snp).

Pyrosequencing (paper III)

Genomic DNA (500 ng) was modified with sodium bisulfite with the EpiTect Fast DNA Bisulfite Kit, according to the manufacturer's protocol (Qiagen, Valencia, CA). Two PCR assays were performed to amplify a total of 15 CpG sites in the genomic *WT1* sequence, located upstream of the transcription start site (UTSS) and in the *WT1* 5'-UTR, as described in paper III. Real-time sequencing was performed with the PyroMark Q24 advanced system (Qiagen). Data was analyzed with PyroMark Q24 1.0.10 Software (Qiagen), and the percentage of methylation (mC/mC+C) was calculated for each CpG site. As a reference, we chose the mean percentage of methylation at each CpG site in corresponding tumor-free samples. Then, we designated two standard deviations of the mean value from the tumor-free samples as a cut-off level for each CpG site. When the methylation level of one CpG site in a tumor sample was above the cut-off level, the sample was considered hypermethylated at that specific CpG site.

Transient transfection (paper IV)

siRNA transfection

Transfections were performed with the pooled siGENOME SMART pool of *WT1*, *p63*, and *p53* siRNAs (Dhamacon, Chicago, USA). To suppress expression of *WT1*, *p63*, and *p53*, we transiently transfected FaDu cells with siRNAs that targeted transcripts of *WT1* (12.5 nM/well), *p63* (5 nM/well), and *p53* (5 nM/well) in six well plates (3×10^5 cells/well) and in 96-well plates (8×10^3 cells/well). Transfections were performed with lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) to suppress gene expression. Cells were harvested at 24, 48, or 72 h after transfection for further analysis.

WT1D plasmid transfections

To induce overexpression of the WT1D isoform, pcDNA 3.1 (+) vectors (Invitrogen, Carlsbad, CA, USA) that carried the *WT1D* variant were constructed, as previously described [164]. FaDu cells were transiently transfected with 3 µg *WT1D* pcDNA 3.1 (+) per well and lipofectamine 2000 (Invitrogen) in six-well plates (5×10^5 cells/well).

MTT assay (paper IV)
The Vybrant MTT Cell Proliferation Assay Kit (Invitrogen) was applied to measure cell proliferation. FaDu cells were collected at 0, 24, and 48 h after transfection and labeled with the MTT solution (3-(4.5-dimethyldiazol-2yl)-2.5-diphenyltetrazolium bromide) mixed with SDS-HCL. Absorbance was measured on a spectrometer at 570 nm.

Western blot (paper IV)
Total protein was extracted with lysis buffer (0.5% NP-40, 0.5% NA-DOC, 0.1% SDS, 150 nM 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 with the BCA reagent (Thermo Scientific, Rockford, IL, USA). Samples (20 µg) were separated on a 10% SDS polyacrylamide gel by electrophoresis (BIO-Rad, Hercules, CA, USA); then, proteins were transferred to a PVDF membrane (Millipore, Billerica, MA, USA). The membrane was blocked with TBST, which contained 5% non-fat dry milk. Then, the membrane was incubated with mouse-monoclonal antibodies against WT1 (1:250, DAKO, Glostrup, Denmark), p63 (1:2000, DAKO), p53 (1:1000, Abcam, Cambridge, UK), and β-actin (1:10000, Millipore). Next, membranes were incubated with peroxidase conjugated anti-mouse polyclonal antibodies (1:5000, DAKO). Proteins were visualized with a chemiluminescent detection system (ECL-advanced, GE healthcare UK) in a ChemiDoc XRS (Bio-Rad, Italy).

Chromatin immunoprecipitation (ChIP)/PCR analysis (paper IV)
ChIP analysis was performed with the Chromatin Immunoprecipitation Kit (Upstate Millipore, Billerica, MA, USA), according to the manufacturer's instructions. The SKOV3 cell line, derived from the ascitic fluid of a woman with an ovarian tumor (ATCC HTB-77), had no endogenous WT1 expression and no p53 expression (p53 mutations at codons 89 and 179); this cell line was used as an extra negative control [129, 165]. Anti-WT1 antibody (C-19, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) or normal rabbit IgG (Cell Signaling technology Inc, Danvers, MA, USA) was used for immunoprecipitation. PCR amplification was performed with the immunoprecipitated DNA or with input chromatin. The PCR conditions and the primer sequences for *p63* promoters are detailed in paper IV. PCR products were fractioned on a 1% agarose gel, and stained with ethidium

bromide. Stained DNA was visualized with an Ultraviolet Transilluminator (Spectroline, Westbury, NY, USA). For quantitative real-time PCR, we used the SYBR green master mix (Bio-Rad). For PCR amplification of cDNA, we used the IQ Sybr Green supermix (Bio-Rad), and samples were analyzed on an Iq5 (Bio-Rad).

Genome-wide gene expression array (paper IV)

From each sample, 200 ng RNA was used to produce biotinylated cRNA with the 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 with the Illumina iScan Reader. The Genome Studio (Illumina) software was used for data processing. For normalization, background correction, and variance stabilization, we used the transformation Lumi package [166]. Differentially expressed genes were identified based on a moderated t-test with the MEV software package from TIGR [167]. Network analysis was carried out with Metacore software (GeneGo Inc, St Joseph, MI, USA). Pathway analysis was carried out with the Database for Annotation, Visualization, and Integrated Discovery (DAVID) tool [168].

Genome-wide DNA methylation array (paper III)

DNA samples were converted with bisulfite with the EZ-96 DNA Methylation^{TM} Kit (Zymo Research), according to the manufacturer's protocol. Bisulfite-converted DNA was submitted to genome-scale DNA methylation profiling with the Illumina Infinium Human Methylation450 BeadChip platform (Illumina Inc., San Diego, USA), which allows analysis of 485,577 CpG sites. Approximately 200 ng DNA was applied to each array. BeadChip arrays were handled according to the manufacturer's protocol (Illumina Inc.) and scanned with an iScan SQ instrument (Illumina Inc.). Fluorescence intensities from the BeadChips were evaluated with the Methylation module (1.9.0) in the Genome Studio software (V2011.1). We preprocessed the methylation array data by omitting CpG sites with three or fewer reported beads/array, CpG sites with a detection *P*-value greater than 0.05, and CpG sites located within ten base pairs of a known SNP. *WT1* was represented by 58 CpG sites in the array, and after preprocessing, 53 remained for further analysis. Methylation levels were determined according to the definition supplied in the Genome Studio software (Illumina Inc.), which calculated the ratio (β-value) between the fluorescence intensity from

the methylated alleles and the total intensity; this ratio gave rise to a number between 0 (completely unmethylated) and 1 (completely methylated). β values were then normalized with the BMIQ method to compensate for the two different bead types used in the methylation array [169].

Protein co-immunoprecipitation (paper IV)

FaDu cells were lysed in cold lysis buffer (0.5% NP-40, 0.5% NA-DOC, 0.1% SDS, 150 nM NaCl, 50 mM Tris pH 7.5, 1 mM EDTA, 1 mM NaF) supplemented with protease inhibitor (Sigma-Aldrich, St. Louis, USA). Equivalent amounts of protein lysate were incubated with the anti-WT1 (catalog no. M3561, DAKO, Glostrup, Denmark) and anti-IgG (catalog no. 2729S, Millipore, Billerica, U.S.A.) antibodies at 4°C overnight. Samples were then incubated with Protein G Sepharose 4 Fast Flow (GE Healthcare, Uppsala, Sweden). Immuno-blotting was conducted to analyze immunoprecipitated proteins with anti-WT1 (1:250, DAKO, Glostrup, Denmark), anti-p53 (1:2000, Abcam, Cambridge, UK), and anti-p63 (1:2000, DAKO, Glostrup, Denmark) antibodies.

Statistical Analysis

Statistical analysis was performed with SPSS (version 18 or 19) statistical software (SPSS Inc., Chicago, IL, USA). The Mann-Whitney U test was used to compare differences between two independent variables. Fisher's exact test (when the sample size was <5) was used for comparing proportions. The Kruskal-Wallis one-way analysis of variance was used for group comparisons. Correlations between 2 variables were tested with Spearman correlation tests. The χ^2 test was used to determine the significance of observed differences in proportions (papers II and III). The Kaplan-Meier method was used to estimate the distribution of freedom from relapse (FFR), the OS, and the disease specific survival (DSS) (papers I-III). The log-rank test was used to determine differences in the probability of FFR and survival between groups. For patients that achieved CR, FFR was measured from the date of diagnosis until the day of disease relapse. DSS was defined as the time period from diagnosis to death from the disease or to the last follow-up. OS was calculated from the time of diagnosis to the date of death or last follow up. *P*-values < 0.05 were considered significant.

Results and Discussion

Paper I

Reduction in *WT1* gene expression during early treatment predicts the outcome in patients with acute myeloid leukemia

Correlation of WT1 transcript levels normalized against two different control genes

We compared the usefulness of the *β-actin* gene and the *ABL1* gene as CGs for normalizing *WT1* expression levels in a total of 202 samples, including 48 diagnostic and 154 follow-up samples. RQ-PCR with TaqMan technology was performed to quantify the gene expression levels. *WT1* transcripts were detectable in 133 of 202 samples. We found significant agreement in *WT1* gene expression levels normalized with either the *β-actin* gene or the *ABL1* gene ($r = 0.96$, $P < 0.001$).

In previous MRD studies, the *β-actin* gene was used as the CG for normalizing *WT1* expression [92, 170, 171]. It has been suggested that *β-actin* transcripts were probably affected less than other genes by chemically-induced or stress-induced damage to gene activity [51, 172]. However, the *β-actin* gene was shown to have pseudogenes, which could cause overestimation of gene expression [173, 174]. Another argument against *β-actin* as a CG in patients with AML was the fact that monosomy 7 occurs in 4% of patients with AML, and the functional *β-actin* gene is located on chromosome 7 [175]. More recently, several studies have used the *ABL1* gene as a CG for normalizing *WT1* transcripts [53, 151, 176]. Beillard et al. identified several suitable CGs for diagnosis and detection of MRD in patients with leukemia [176]. However, only *ABL1* was proposed for use as a CG, because its mRNA expression was not significantly different in normal and leukemic samples at diagnosis. Another study showed that some primers for *ABL1* could amplify *ABL1* transcripts from translocations in Philadelphia-positive leukemia [177]. Other studies showed that *β-glucuronidase* would be an optimal CG for molecular monitoring of chronic myelogenous leukemia [178, 179]. However, a strong correlation was demonstrated among *WT1* gene transcription values, when *β-actin*, *ABL1*, and *GAPDH* were used as CGs for normalization [173].

Prognostic significance of reductions in WT1 gene expression during treatment

To determine the prognostic relevance of *WT1* gene expression levels in both BM and PB samples with regard to OS and FFR during follow-up, different intervals were evaluated, as follows. Interval 1: <1 month, samples were acquired between 3 and 4 weeks after diagnosis (median 25 d, range 19 to 30 d); and interval 2: samples were acquired between 1 and 6 months (median 2.7 mo, range 1.03 to 5.7 mo). We analyzed the effects of a ≥1-log reduction and a ≥2-log reduction in *WT1* mRNA expression. We found that, in interval 1, the achievement of ≥1-log reduction in BM, *WT1* was associated with improved OS and FFR, when *β-actin* was used as the CG ($P = 0.004$ for OS and $P = 0.010$ for FFR). PB samples could not be analyzed, because very few follow-up samples were available from this interval. In interval 2, the achievement of both ≥2-log and ≥1-log reductions in PB, *WT1* were associated with improved OS and FFR, when both *β-actin* and *ABL1* were used as CGs.

Despite the limited number of patients in this study, our observations were consistent with previous studies, which showed that early MRD measurements could provide predictive information on patient outcome [151, 180]. *WT1* gene expression analysis in PB samples is likely to be the most informative measure, given the low background level of *WT1* in PB relative to BM samples. The study also showed that a combination of two CGs could yield more informative results, but larger patient numbers would be necessary to confirm these data.

Paper II

Single nucleotide polymorphisms in *WT1* in clear cell renal cell carcinoma

Frequencies and features of WT1 SNPs in patients with ccRCC

Sequence analysis was performed on all 10 exons in *WT1* in 182 ccRCC tumor specimens. A total of six different SNPs were identified that were associated with the *WT1* gene. The genotypes of each SNP met Hardy-Weinberg equilibrium. One or two copies of the minor allele were found in exon 1 in 95 tumor specimens, in exon 7 in 52 specimens, and in exon 10 in 27 specimens. The minor allele frequencies of these SNPs were 16.8% for rs2234582 in exon 1; 16% for rs16754 in exon 7; 13.7% for rs1799925 in

exon 1; 7.7% for rs5030315 in exon 10; 6.6% for rs2234583 in exon 1; and 0.6% for rs2234581 in exon 1. Similar frequencies of these SNPs (except for rs2234581) were found in 78 corresponding tumor-free renal cortical tissue samples.

Furthermore, when SNP genotypes were compared between 78 paired tumor and corresponding tumor-free specimens, a high concordance (95%) was demonstrated. We also identified a novel heterozygous missense mutation in exon 1 at nucleotide position 536 (C>A) in only one patient. Previously reported missense, nonsense, or frame-shift mutations in *WT1* hot spots in leukemia or in Wilms' tumor were not identified in the present study [98, 105]. Infrequent mutations in the *WT1* gene were reported in a Japanese study that comprised 34 primary urinary tract cancers in 22 patients with RCC [181]. These results implied that common *WT1* gene mutations are not involved in RCC.

No correlation between WT1 mRNA level and SNP genotypes

We analyzed *WT1* mRNA levels with RQ-PCR in a total of 115 tissue samples, including 100 tumor and 15 tumor-free specimens. Significantly lower mRNA level was observed in ccRCC samples compared to tumor-free renal cortical tissue ($P = 0.001$). This finding was similar to findings in our previous study on *WT1* mRNA level in ccRCC. Those results indicated that down-regulation of *WT1* acted as a tumor suppressor in ccRCC [13]. We then compared *WT1* RNA expression between groups with different *WT1* SNP genotypes. We found no difference in *WT1* mRNA levels between groups in tumors ($P = 0.726$) or in tumor-free tissue samples ($P = 0.779$). This result suggested that the mechanism of *WT1* down-regulation in ccRCC may not arise from any specific *WT1* SNP genotypes. Other mechanisms, like DNA methylation or related oncogenic pathways, may be involved in the reduction of *WT1* expression in ccRCC.

Homozygous minor allele for WT1 SNPs were associated with favorable clinical outcome

We investigated the prognostic impact of the *WT1* SNPs in our entire cohort of 182 patients with ccRCC. The cohort had a median OS of 49.5 months (range 1–300 months) and a median DSS of 49 months (range 1–293 months). To determine whether outcome was associated with genotypes, we

performed separate analyses in patients homozygous for the *WT1* wild-type alleles, those heterozygous, and those homozygous for the minor allele. We found that patients homozygous for the minor allele had longer OS and DSS than patients heterozygous for the minor allele (P = 0.020 for OS and 0.018 for DSS) and those homozygous for the wild-type allele (P = 0.029 for OS and DSS). In addition, we compared patients with different exon 1 *WT1* genotypes in a Kaplan-Meier analysis. Patients homozygous for the minor allele in exon 1 were observed to have a favorable outcome for both OS and DSS compared to patients heterozygous (P = 0.026 for OS and P = 0.022 for DSS) and those homozygous for wild-type *WT1* (P = 0.012 for OS and P = 0.010 for DSS). A subgroup analysis of the rs16754 SNP showed that patients homozygous for the minor allele had a significantly more favorable OS (P = 0.036), and they showed a trend toward a longer DSS (P = 0.060), compared to those with heterozygous genotypes. Unfortunately, too few patients were homozygous for the minor allele; therefore, we were not able to evaluate whether the prognostic impact was independent in a multivariate analysis.

Similar to our results, other studies have recently demonstrated that the minor allele of SNP rs16754 had a favorable effect on OS and relapse-free survival in patients with AML [106, 107, 115, 116]. These findings may be explained by the fact that the rs16754 SNP replaces the rare codon, CGA (6.2 per thousand), with the more frequently used codon, CGG (11.4 per thousand, frequencies obtained from the Codon Usage Database)[182]. This substitution of a rare codon for a more frequently used codon leads to increased translation kinetics, which could potentially affect protein function, as previously demonstrated *in vitro* in E. coli [183]. In contrast to the collected data that showed rs16754 was a positive prognostic factor, other studies demonstrated that it had no significant impact in AML [148, 184].

Paper III

Prognostic Significance of Hypermethylation in the Promoter Region of the Wilms' Tumor Gene 1 in Clear Cell Renal Cell Carcinoma

WT1 DNA methylation detected with the methylation array and pyrosequencing in ccRCC

We performed a genome-wide DNA methylation array on 117 ccRCC tumor specimens and 10 corresponding tumor-free tissue specimens. In the present study, we analyzed the data with a focus only on the *WT1* gene. Our findings

in regions upstream of the transcription start site (UTSS) were of particular interest in considering methylation effects on *WT1* expression. There were 12 CpG sites in the UTSS, 5'-UTR, and exon 1 regions. Significantly high *WT1* methylation levels in 10 CpG sites were detected in tumor specimens compared to adjacent tumor-free tissue samples ($P < 0.01$). CpG site cg22975913, located at -648 bp, was identified in 43 out of 117 patients (37%); this was the most frequently methylated CpG site.

Furthermore, we performed a validation analysis with pyrosequencing in 33 paired tumor and corresponding tumor-free renal cortical tissue samples from patients with ccRCC. We evaluated DNA methylation on CpG sites around the specific locus of the cg22975913 site and in the 5'-UTR region. In the UTSS region, eight CpG sites were sequenced. Significant differences between tumor and tumor-free samples were found in six CpG sites ($P < 0.05$ or $P < 0.01$), including cg22975913 ($P < 0.05$). Among all the CpG sites analyzed, the highest DNA methylation level was detected by pyrosequencing at the cg22975913 site. We found that the frequency of patients with hypermethylation at the cg22975913 site was higher when based on pyrosequencing results (45%) than when based on DNA methylation array results (37%). In the *WT1* 5'-UTR region, we assessed seven CpG sites located at +21 bp to +76 bp close to the transcription start site by pyrosequencing. Methylation between tumor and tumor-free samples was significantly different in six of these CpG sites. The frequency of patients that showed hypermethylation in this region varied from 18% to 36%.

Hypermethylation in the *WT1* gene promoter was previously reported in several different types of cancer. One early study used southern hybridization and found hypermethylation in the promoter and first exon of the *WT1* gene in 25% of patients with primary breast tumors [127]. In a methylation-specific PCR assay, Loeb et al. found *WT1* promoter methylation in 6 of 19 (32%) primary breast tumors [2]. In Wilms' tumor, methylation was detected in the enhancer region in about 33% of tumors [128]. The *WT1* and *WT1-AS* were found to be significantly methylated in 88% of ovarian clear cell adenocarcinomas, but only about 20% of serous adenocarcinomas [129].

Interestingly, when we compared results on methylation at the cg22975913 site obtained with either the methylation array or the pyrosequencing assay in 33 ccRCC tumor samples, we found a strong correlation between the two methods ($r = 0.921$, $P < 0.001$).

The methylation array is often used to detect genome-wide DNA methylation, and pyrosequencing is recommended for validation of the array analysis results. Potapova et al. showed high sensitivity for detecting low levels of methylation with pyrosequencing [185]. Similarly, Håvik et al. reported that 26% of low-grade and 37% of high-grade gliomas were methylated in the promoter of the O^6-methylguanine-DNA methyltransferase, based on MSP, and that hypermethylation was detected in 97% of low-grade and 55% of high-grade gliomas, based on pyrosequencing [186]. Other studies have shown that 6% of the Illumina 450K microarray probes were cross-reactive; thus, these probes could hybridize to alternate sequences that were highly homologous to the intended targets [187, 188]. The authors suggested that another method with high specificity and sensitivity should be used to validate the results, and they strongly recommended pyrosequencing.

Correlation between WT1 mRNA level and DNA methylation in the WT1 promoter in ccRCC tumor samples

RQ-PCR detected *WT1* expression in 32 tumor samples and 18 corresponding tumor-free tissue specimens. Patients with ccRCC were divided into subgroups, based on the methylation status of tumor specimens; the two groups comprised those with and without *WT*1 hypermethylation. In the 5'-UTR sites, hypermethylated tumor samples showed a trend towards significantly lower *WT1* mRNA levels compared to non-hypermethylated tumor samples ($P = 0.076$). However, no significant difference between groups was found in corresponding tumor-free tissues ($P = 0.678$). In the UTSS region, no difference in *WT1* mRNA expression was observed between hypermethylated and non-hypermethylated groups, either among tumor samples ($P = 0.386$) or among corresponding tumor-free tissues ($P = 0.122$).

We also compared *WT1* mRNA expression in different subgroups, based on cg22975913 methylation status in tumor and tumor-free tissues. Samples were assigned to subgroups based on a cut-off of 20% methylation; those

above the cut-off were considered hypermethylated and those below the cut-off were considered non-hypermethylated. No differences in *WT1* mRNA expression were observed between hypermethylated and non-hypermethylated subgroups of tumor samples (P = 0.132) or the corresponding tumor-free tissues (P = 0.556).

Several previous studies also found no correlation between methylation in the *WT1* promoter region and gene expression. In colorectal cancer, Hiltunen et al. reported that CpG sites within the *WT1* promoter region were methylated in adenomas and carcinomas, but the level of *WT1* gene expression was not different between normal colonic mucosa and malignant carcinoma [125]. However, one study showed that methylation of the CpG islands associated with the *WT1* promoter was correlated with gene silencing in primary breast tumor and in several breast cancer cell lines [2].

Considering the heterogeneity of cell subpopulations in ccRCC tumor tissues, we analyzed the *WT1* methylation status in two ccRCC cell lines (786-O, with WT1 protein expression, and A498, without detectable WT1 protein expression) and two ovarian cancer cell lines (OVCAR-3, with WT1 protein expression, and SKOV-3, without detectable WT1 protein expression). The results showed high methylation levels in both the *WT1* UTSS promoter and the 5'-UTR regions in A498 and SKOV-3 cell lines, but low levels of methylation in 786-O and OVCAR-3 cell lines. These findings suggested that *WT1* methylation in the UTSS promoter and 5'-UTR region was correlated with gene silencing in these cancer cell lines.

Prognostic significance of WT1 methylation status

Based on the methylation array results in the patient cohort, no associations were identified between methylation and age, sex, tumor grade, tumor stage, tumor size, OS, or DSS. However, based on pyrosequencing analysis in the same patient cohort, hypermethylation was found to be related to patient age (P = 0.037), but not to other characteristics, including sex, tumor grade, tumor stage, tumor size, OS, or DSS.

Furthermore, we performed survival analysis in 117 patients with ccRCC to determine whether methylation of the CpG site, cg22975913, was related to survival. The results from the methylation array analysis showed no significant difference in outcome between patients with and without hypermethylation (P = 0.617 for OS and 0.949 for DSS). However, a survival analysis in 33 patients with ccRCC based on pyrosequencing

showed that patients with hypermethylation at the cg22975913 site had significantly worse OS (P = 0.035), and they showed a trend towards worse DSS (P = 0.053), compared to those without cg22975913 hypermethylation. Interestingly, a subgroup analysis in patients with stages I-III ccRCC showed that hypermethylation in the cg22975913 site was significantly correlated to shorter OS and DSS (P = 0.020 for OS and 0.019 for DSS), but no prognostic impact was found in patients diagnosed with stage IV ccRCC (P = 0.355 for OS and DSS).

This might be explained by a generally poor survival in patients with stage IV disease; their 5-year cancer-specific survival rate was only 20% [189]. However, previous studies on *WT1* methylation performed in patients with colorectal cancer, breast cancer, Wilms' tumor, ovarian cancer, and lung cancer have not demonstrated any impact on patient outcome [2, 125, 128-130].

Paper IV

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

Altered cell proliferation with knockdowns of WT1, p63, and p53

The FaDu cell line (ATCC HTB-43), derived from hypopharyngeal squamous cell carcinoma, was used for transfection experiments. MTT assays were performed to determine the effect of WT1, p63, and p53 on cell proliferation in FaDu cells. Knockdown of WT1 resulted in a significant decrease in cell proliferation at 24 and 48 h after transfection ($P < 0.05$). Similarly, silencing *p63* RNA induced a considerable decrease in cell proliferation at both time points ($P < 0.05$). It has been shown that p53 function is inactivated in up to 80% of SCCHN cases [190]. In the FaDu cell line, p53 has a point mutation at codon 248 (Arg→Leu) [191]. This R248L mutation in p53 does not completely abolish its inhibitory effect on cell proliferation in this cell line. We found that, when p53 was knocked down in these cells, a significant increase in cell proliferation was demonstrated at 48 h after transfection compared to control cells ($P < 0.05$). Our results supported the expected oncogenic role of the *WT1* and *p63* genes in this cell line.

In several other types of cancer cells, including non-small cell lung cancer and several solid cancer cells, increased cell proliferation was induced by WT1 [192, 193]. The WT1D isoform was recently found to induce cell proliferation in oral squamous cell carcinoma cells [194]. The *p63* gene was found to be overexpressed in a majority of patients with squamous cell carcinomas and SCCHN [195]. In FaDu cells, ΔNp63 was found to be the main p63 isoform [6, 31]. One previous study showed that knockdown of the ΔNp63 isoform, but not the TAp63 isoform, inhibited cell proliferation in some SCCHN cell lines [196]. However, another study showed that silencing ΔNp63 in FaDu cells did not alter the proliferation state, as judged by Ki-67 expression and FACS analysis of cell cycle phase DNA content [160].

Correlation between WT1 expression and p63/p53 in FaDu cells

To study the relationship between WT1 and p63/p53 in SCCHN, we performed transfection experiments in FaDu cells. We suppressed expression of WT1, p63, and p53 with siRNA technology. Western blot analyses showed that we successfully silenced the *WT1* RNA, which resulted in downregulated expression of the WT1 protein. Cells with suppressed WT1 expression showed distinctly reduced expression of ΔNp63 (68 kDa) compared to control cells. However, the expression of TAp63a (75 kDa) was much weaker than ΔNp63. We observed no changes in TAp63α expression in our experiments. WT1 knockdown cells also showed a slight decrease in p53 protein expression at 72 h after transfection. When p63 was knocked down, a slight decrease in WT1 protein expression was observed at 48 and 72 h after transfection.

Also, a reduction in p53 expression was observed at 72 h after transfection. However, when p53 was knocked down, no alterations in WT1 or p63 protein expression were observed. To confirm the positive correlation between WT1 and ΔNp63, we performed an additional experiment, where a plasmid that carried the WT1D variant was transfected into FaDu cells. In cells with forced overexpression of WT1D, we observed upregulation of ΔNp63 protein levels. These results indicated 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

To assess whether *p63* was a target gene of WT1, we used ChIP/PCR to examine the binding properties of WT1 to the *p63* promoters. Two putative GNGNGGGNG WT1-binding sites in the *TAp63* promoter and one putative WT1-binding site in the *ΔNp63* promoter were identified in a sequencing analysis. ChIP was performed with FaDu cells that were either transfected or not transfected with WT1D. The chromatin was precipitated with WT1 antibodies. PCR amplification could be demonstrated in the region of the second WT1-binding site in the *TAp63* promoter and at the *ΔNp63* WT1-binding site. These findings indicated that the WT1 protein could directly bind to the promoters of the two main p63 isoforms, TAp63 and ΔNp63. However, the WT1 binding site, 341 (P1, -502 to -493), which lies far from the major transcription start site in the *TAp63* promoter, was not involved. We did not find any alterations in TAp63 expression in our experiment. This low efficiency may be explained by the very low expression of TAp63 in FaDu cells detected on western blots and by the fact that only one binding site for the WT1 protein was found in the *TAp63* promoter by ChIP/PCR. As mentioned previously, ΔNp63 is the only major isoform expressed, and this isoform plays a major functional role in FaDu cells.

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

Genes that showed altered expression in response to knocking down WT1 or p63 were detected with microarray analysis. Silencing *WT1* RNA induced significant fold-changes in 848 genes compared to control. In cells with suppressed p63 expression, significantly altered expression was found in 925 genes. Interestingly, when we combined the two profiles, we found that 124 genes had significantly altered fold-changes ($P < 0.05$). Eighteen of these genes were involved in cell proliferation, cell cycle regulation, and DNA replication (Table 3). Moreover, five of these genes were previously described as p63 target genes. ΔNp63 has been reported to directly repress expression of p53-target genes, *IGFBP-3 [197]* and *SFN* (*14-3-3σ*) [198]. *CITED2* and *Skp2* were also identified previously as target genes of p63 [199, 200]. *CDKN1B* ($p27^{kip1}$) expression has been shown to be inversely correlated to Δ*Np63* expression, which suggested the possibility that ΔNp63 may directly negatively regulate *CDKN1B* transcription [196]. The fold changes in expression were nearly identical in 11 of these 18 genes. Therefore, it was unlikely that WT1 regulated these genes through indirect regulation of p63 target genes. According to immunoblot results, WT1-

knockdown cells expressed p63 at reduced levels, which reduced transcriptional regulation, in contrast to the absence of transcriptional regulation observed in p63-knockdown cells. Distinct differences in the expression of *MMP7*, *RARRES1*, *C13orf15*, and *CITED2* genes were observed 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. WT1 might indirectly regulate those genes. *CITED2*, as mentioned previously, was the only known p63 target gene of the above listed genes [199].

In addition, with a Metacore GeneGo analysis, six known WT1 target genes and 27 known p63 downstream target genes were found to be affected in WT1 knockdown cells. These genes are known to be involved in cell cycle, cell growth, cell migration, cell proliferation, inositol phosphate metabolism, and pyrimidine metabolism. *SFN* was previously found to be negatively regulated by ΔNp63 in primary human epidermal keratinocytes (HEKs), as described above [198]. *Skp2* expression was found to be positively regulated by p63 in HEKs [200]. With a ChIP-on-chip array analysis, Huang et al. found that the ΔNp63 protein could bind to the *CAD* promoter in squamous cell carcinoma cells, when cells were exposed to cisplatin [201]. A previous study showed that p63 could activate the *CITED2* promoter in keratinocytes [202]. In the human keratinocyte (HaCaT) cell line, TAp63 was found to activate *GDF15* by directly binding to the promoter [203]. The proapoptotic protein, IGFBP-3 was shown to be negatively regulated by ΔNp63α in squamous epithelial cell lines, HaCaT and SCC-1 [197]. However, these known p63 target genes were not reported to be correlated with WT1 expression. Further studies are needed to determine whether WT1 can directly regulate these genes.

WT1 protein interacts with p53, but not p63

To study the protein interaction between WT1 and p53/p63, we performed a co-immunoprecipitation analysis. We detected p53 in WT1 immune-complexes, but not p63. This finding indicated that a protein interaction occurred between WT1 and p53 in FaDu cells. Previous studies have presented evidence for a protein-protein interaction between WT1 and p53 in BRK cells, and in Wilms' tumor [88, 204]. This interaction was not abolished by a p53 mutation in BRK cells, at a position homologous to human codon 248. Furthermore, WT1-induced p53 protein stabilization has been reported in Saos-2 cells [16].

Table 3. Significant fold-changes in the expression of genes involved in cell proliferation, cell cycle regulation, and DNA replication by knocking down WT1 or p63 in FaDu cells

Term	Gene name	Expected effect*	Fold change (vs. control)	
			WT1 siRNA	**p63 siRNA**
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.

High WT1 mRNA expression in clinical samples

WT1 mRNA levels were analyzed by RQ-PCR 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 ($P < 0.001$) and normal control tongue tissues ($P = 0.001$). This finding indicated that *WT1* was overexpressed in SCCHN. In support of our result, Oji et al. also reported overexpression of *WT1* in SCCHN tissue samples [157]. No significant correlation was found between *WT1* mRNA levels and clinical features, including age, sex, tumor stage, OS, or DSS in our limited patient cohort. The potential prognostic impact should, however, be studied in larger patient cohorts.

Conclusions

Paper I
A good correlation was observed between *WT1* expression levels normalized with two different CGs (*β-actin* and *ABL1*). A reduction in *WT1* expression in bone marrow (≥ 1-log) detected less than 1 month after diagnosis could predict outcome when *β-actin* was used as control, and a reduction in *WT1* expression in peripheral blood (≥ 2-log) detected between 1 and 6 months after treatment start could predict outcome, irrespective of the CG used. These data suggested that the analysis of *WT1* expression may be a useful tool for monitoring MRD in AML.

Paper II
Six different SNPs in the *WT1* gene were identified, and at least one or two copies of the minor allele were found in 61% of RCC tumor samples. No correlation was observed between *WT1* SNP genotypes and mRNA expression levels. None of the previously reported *WT1* mutations in leukemia were found in RCC. However, we found favorable outcomes associated with homozygosity in the SNP that represented the minor allele of *WT1*.

Paper III
The CpG site, cg22975913, in the *WT1* promoter region was identified as the most frequently hypermethylated CpG site in both methylation array and pyrosequencing analyses. A trend was shown towards a significant correlation between *WT1* mRNA levels and hypermethylation in the 5'-UTR region. Furthermore, hypermethylation in the *WT1* promoter region was found to be associated with patient age and with a worse prognosis in patients with ccRCC.

Paper IV
A novel positive correlation was found between *WT1* and *p63* gene expression, and this correlation confirmed that WT1 regulated *p63* expression through direct binding to the *p63* promoters. Both WT1 and p63 were found to promote cell proliferation in SCCHN cells. Furthermore, *in vitro* experiments showed that the expression levels of 18 genes involved in cell proliferation, cell cycle regulation, and DNA replication were altered by silencing *WT1* and *p63* RNAs. Several known WT1 and p63 target genes were affected by knocking down WT1. Additionally, *WT1* mRNA levels were overexpressed in SCCHN samples.

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