2. INTRODUCTION

2.1 WT1 Wilms' Tumor Gene 1

Wilms Tumor Gene 1 (WT1) is a potent transcription factor that appears to play a joker role in cell growth, differentiation and apoptosis. WT1 upregulation and lack of expression – depending on the tissue and the time point of deregulation - might both lead to the development of neoplasia. The following work focuses on aspects of upregulation of WT1 expression by WT1 activators and its impact on human neoplasia, therefore the aspects of deficiency of WT1 expression in human neoplasia are omitted.

2.1.1 Characteristics of WT1 gene product

WT1 gene lies on the locus 11p13. WT1 is a typical transcription factor consisting of two domains: a zinc-finger DNA binding domain and rich in proline and glutamine regulatory domain (Call et al., 1990; Gessler M et al., 1990; Morris et al., 1991). WT1 transcript is subjected to alternative splicing at two main sites: position I splice is the exon 5 insertion and position II splice is three amino acids insertion between the third and fourth zinc finger (+KTS splice)(Haber D.A. et al., 1991). Splicing at the position I may have influence on transcriptional activity of WT1 – exon 5 positive variants tend to have higher activity than those lacking exon 5 (Wang Z.Y. et al., 1993). Splicing at the position II has strong effect on target sequence recognition and intracellular localization of WT1: -KTS variant is found in the areas of transcriptional activity and +KTS variant is associated with splicing factors (Bickmore W.A. et al., 1992; Larsson S.H. et al., 1995).

2.1.2 Regulation of WT1 Expression

Physiological WT1 expression is restricted only to few types of cells and tissues. The promoter region of Wilms tumor gene shares similarities with

suppressor genes promoters such as Rb and P53 genes – it is a member of a family of GC rich, TATA-less, CCAAT-less polymerase II promoters (Fraizer G.C. et al., 1994, Hofmann W. et al 1993). The other regulation elements of WT1 expression are enhancers – one located 50kb 3' downstream from the promoter second localized inside the exon 1. The regulating sequences of WT1 contain binding motifs for PAX2, PAX8, Sp1,
NF-κB, IL6, PEA3 and WT1, among other transcription factors. The PAX2 and PAX8 factor binding sites are situated in promoter regions and GATA1 sites are localized in promoter and enhancer regions.

2.2 PAX2

PAX2 gene encodes a transcription factor containing a paired box DNA binding domain, which binds to GTTCC sequence motif (Dressler G.R. and Douglas D.C., 1992). It is a member of Paired-boX (PAX) gene family that includes 9 proteins that are implicated in control of the tissue differentiation. PAX2 controls the urogenital differentiation (Dahl W. et al., 1997). The role of Pax2 is an activation of WT1 promoter and possibly interaction with WT1 protein (Dehbi M. et al., 1997). The complexes of WT1 and PAX2 were shown to form spontaneously in vitro and were found in the developing kidney tissue (Discenza T.M. et al., 2003). On the other side, WT1 is negative regulator of PAX2 expression as it directly binds to its promoter during nephrogenesis and blocks PAX2 expression (Ryan G. et al., 1995). The coexpression and co localization of these two proteins was also shown in normal and cancer breast tissue, where it seems to be connected with progesterone dependent mammary growth (Silberstein G.B. et al., 2002). PAX2 promoter has been well characterized (Stayner C.K. et al., 1998). It contains one transcription start site,
is GC rich and lacks consensus sequences of TATA and CCAAT box. PAX2 and WT1 promoters both belong to a subclass of polymerase II promoters, and are not housekeeping genes promoters. The WT1 binding sites present in PAX2 promoter are highly conserved across mouse and human species. PAX2 with other PAX family genes are frequently expressed in human neoplasia, and RNAi of PAX2
in the expressing cell lines leads to induction of apoptosis in ovarian and bladder cancer lines (Muratovska A. et al., 2003)

2.3 PAX8

PAX8 is another member of Paired box gene family being able to activate the expression of WT1 (Dehbi M. and Pelletier J., 1996). Physiologically Pax8 is expressed in the developing kidney mesenchyme where its expression peaks prior to onset of WT1 expression. There are two conserved TGCCC - PAX8 binding sites in WT1 promoter in human and mouse species, by which PAX8 can activate WT1 expression (Zannini 1992). So far there is no direct evidence that PAX8 dependent activation of WT1 may be important in the development of human neoplasia – the indirect clue comes from primary thyroid cancers (Oji Y. et al., 2003) where the PAX8 and WT1 are being expressed at high proportion of tumors. It is also known that PAX8 plays a role in the thymocyte differentiation (Lin R.Y. et al., 2003) – but the qualitative and quantitative correlation of PAX8 and WT1 was never explored.

2.4 GATA1

GATA1 is a founding member of the GATA protein family, that consist of set of transcription factors that are essential for erythrocyte differentiation and recognize WGATAR sequence localized in the regulatory elements of the other genes (Weiss M.J. and Orkin S.H., 1995). The expression of GATA1 was found in the multipotential progenitors of hematopoietic cells. Expression of GATA1 is restricted to the erythroid cells at the later stages of differentiation, megakaryocytes, eosinophils and mast cells (Mouthon M.A. et al., 1993, Sposi N.M. et al., 1992, Zon L.I. et al., 1993). GATA1 plays an essential role in the erythrocyte differentiation and failure of its expression leads to the erythrocyte maturation arrest (Weiss M.J. et al., 1994) and lack of platelet synthesis (Shivdasani R.A. et al., 1997). Disruption of the GATA1 promoter that leads to the deficiency of its synthesis causes death of homozygous offspring and severe anemia in heterozygots (Takahashu S. et al., 1997). Absence of GATA1 expression leads inevitably to a lethal anemia (Whyatt D. et al., 2000). On the other hand, expression of GATA1 in erythropoietic precursors hinders their apoptosis (Weiss M.J. et al., 1995). GATA1 interacts with Sp1 – another factor, which was shown to bind to the WT1 promoter (Merika M. and Orkin S.H., 1995). The connection between expression of GATA1 and WT1 is not yet fully understood.

It was shown that GATA1 and WT1 are co-expressed in a large proportion of human AML (Patmasiriwat P. et al., 1999, Siehl et al., 2003). Putatively GATA 1 transactivates WT1 hematopoietic specific enhancer and thus promotes WT1 synthesis in leukemic cell lines (Wu Y.J. et al., 1995). However, activation of WT1 by GATA1 seems
to be restricted to cells that have a proper WT1 enhancer function.

2.5 WT1 expression in leukemia

The first data implicating involvement WT1 in the development of human neoplasia came from leukemia. Its elevated expression was found in acute myeloid leukemia and in blast crisis of chronic leukemias (Miwa H. et al., 1992; Menssen H.D. et al., 1996). As the WT1 was physiologically expressed in the early hematopoietic cells pathogenic function of WT1 was traced to the regulation of early stages
of hematopoietic differentiation. The protein expressed in leukemia was not mutated so it was proposed that WT1 was contributing to the pathogenesis of leukemia
by exerting its physiological function and supporting of the undifferentiated state (Miyagi T. et al., 1993). The targeting and downregulation of WT1 expression with antisense oligonucleotides caused growth inhibition or apoptosis of leukemic cells, but did not inhibit normal CFU-GH colony formation (Yamagami T. et al., 1996, Wu X. et al. 1999). From the begging it was suggested that expressed WT1 could
be a possible marker of leukemic blast cells as its expression was clearly present in non treated patients with AML and with patients at relapse but wasn't in CR patients and healthy controls (Brieger J. et al., 1994, Bergmann L. et al., 1997, Inoue K. et al., 1994). The levels of WT1 that are found in leukemia are at least 10 times higher than the expression in WT1 expressing bone marrow cell subsets (Inoue K. et al., 1997). The elevated expression of WT1 in AML is also associated with worse long-term outcome (Bergmann L. et al., 1997). In the animal model of leukemia WT1 was absent at the pre leukemic stages, and appeared only at the late stages
of leukemogenesis. Moreover, the WT1 expression was accompanied in large proportion of cases by abnormal expression of GATA1 (Osaka M., 1997). Possible mechanism in which WT1 contributes to leukemia is blockage of signaling pathways for differentiation inducing factors - a constitutive expression of the WT1 blocks

differentiation (Svedberg H. et al., 1998). This mechanism is connected with WT1 repressor action and requires DNA binding domain (Inoue K. et al., 1998; Deuel T. et al., 1999).

2.6 WT1 expression in solid tumors

Apart from leukemia WT1 expression was also found in solid tumors (Oji Y. et al., 1999, Oji Y. et al., 2002). WT1 is frequently overexpressed in the *de novo* small cell lung cancers. However, just like in leukemia, the gene is not mutated thus the overexpression of wild type allele is important for cancer pathogenesis. WT1 mRNA in lung cancers is expressed at levels about 100 times lower than in K562 cell line but still the expression is sufficient for protein synthesis. The splicing of WT1 seems to be unaffected as the proportions of the splicing variants are similar in different samples. Therefore it is very likely that the pathogenic changes are situated on the higher levels of gene regulation. The high expression of WT1 was also found in breast cancer patients where it seems to be an independent prognostic marker (Miyoshi Y. et al., 2002). Just like in leukemia also in breast cancers correlation with poor outcome and increase of expression at relapse was found. The downregulation of WT1 expression by antisense oligonucleotides in breast cancer leads to inhibition of tumor cell proliferation (Zapata-Benavides P. et al., 2002).

2.7 Prospects of immune therapy against WT1

Since the beginning of medicine a “magic bullet” is sought that would hit the diseased tissues and leave the healthy tissues intact. The WT1 gene is a promising prospect target for such “magic bullet” approach. WT1 found its way into the spotlight of tumor immunotherapy with a discovery of a CTL response to WT1 antigens in neoplasia (Oka Y. et al., 2000). CTL could lyse cells pushed with WT1 peptides in the HLA-A2.1 restricted fashion. However, they were unable to lyse cells lacking HLA-A2.1 independent on the WT1 expression status. It was soon discovered that such response against WT1 could be elicited in vivo (Gaiger A. et al., 2000). The generation of CTLs specific for WT1 was observed, which were able to lyse WT1

expressing pathologic cells but left intact healthy cells expressing WT1. The WT1 specific antibodies were also found in the sera coming from patients with leukemia that overexpressed WT1. The observation followed that CTL specific for WT1 could lyse leukemic CD34+ progenitor cells (Gao L. et al., 2000). It was also shown that CTLs specific for WT1 could inhibit colony formation by transformed progenitor cells from CML but did not affect colony formation by normal CD34+ cells. It was also shown that WT1 specific CD8 T cells occur spontaneously in patients with AML (Scheibenbogen C. et al., 2002). Therapy directed against WT1 epitopes proved to have curable effect in murine model of leukemia (Oka Y. et al., 2000). The early clinical results demonstrate that immunization against WT1 epitopes can support remission of human AML (Mailander et al., 2004).

2.8 WT1 expression and regulatory sequence methylation

Yet another level of gene expression is a change in the methylation of the promoter regions of genes. It is probably most common epigenetic change that is introduced in the human genome (Jones P.A. et al., 2001) It is known that there are profound changes in promoter methylation in human neoplasia with strong effects on gene expression patterns (Baylin S.B. et al., 1991). They appear to occur at the early stages of tumor genesis (Nass S.J. et al., 2003). The changes in the methylation of WT1 promoter were so far explored in small extent in normal and neoplastic tissue. Physiologically changes of WT1 promoter methylation are a part of a plausible tissue specific regulatory system that restricts expression of WT1 only to paternal allele in human fibroblasts and lymphocytes (Mitsuya K. et al., 1997).
In neoplasia WT1 promoter seems to be a site of frequent methylation changes – hypermethylation of WT1 promoter CpG islands was shown in human breast cancer samples and it was connected with lack of expression of WT1 (Laux D.E. et al., 1999). The contrary evidence for WT1 enhancer was provided by Mares J. et al., 2001 - the enhancer region seems to be methylated in all healthy blood and kidney samples but unmethylated in 2/3 of tumor samples of breast cancer. More sensitive analysis with restriction enzymes and microarrays showed that 25% of breast cancers have hypermethylation of whole WT1 promoter (Chen C.M. et al., 2003). Despite the promoter hypermethylation most of breast cancers express WT1 at high

levels what seems to be an exception form a generally accepted rule that connects hypermethylation with abolishment of gene activity (Loeb D.M. et al., 2001). The methylation changes of WT1 promoter region were also found in the Wilms' Tumors where the hypomethylation and biallelic expression of antisense transcript is observed (Malik K. et al., 2000). The potency of even single site methylation changes was shown in mesothelioma, another tumor with common expression of WT1 gene. Alternation of single tumor specific methylation site in the first exon is responsible for activation or inactivation of WT1 gene (Kleymenova E.V. et al., 1998). Since the promoter of WT1 is not different form other promoters it is the interplay between the transcription factors and tissue specific methylation pattern that is responsible for tissue-restricted expression of WT1.

There is a vast panel of the PCR based methods that can be used for analysis of the methylation status of the CpG residues that were recently reviewed (Liu Z.J. and Maekawa M., 2003). The most of the PCR based methods share disadvantages: need for new primer design, false positive results in methods based on restriction enzymes, need for extra bisulfate DNA modification in pre PCR steps. The approach presented here bases on advantages given by Light Cycler real time quantitative PCR and combining with restriction of the DNA with methylation sensitive enzyme. The sample of DNA is restricted with enzyme and the amount of DNA pre and post digestion are quantified – the difference reflects amount of digested DNA thus unmethylated DNA. Samples of methylated and unmethylated are included as controls for the reaction. So far the quantitative analyses were mostly based on methylation based on bisulfate modification of DNA and analyses of melting curves – the main disadvantage of those methods was need for new primers and bias coming from incomplete bisulfate modification (Worm J. et al., 2001). The approach presented here excludes the need for new primer design and optimization and allows rapid analysis of methylation with virtually any pair of primers.