7. DISCUSSION

Wilms Tumor gene 1 seems to be a new promising target for the therapy of different human neoplasia including acute myeloid leukemia and breast cancers. The position of WT1 in the regulation of growth of neoplastic cells make WT1 an ideal target for future therapeutic intervention. The aspects of regulation of WT1 expression are only partially studied. The series of putative regulators has been targeted basing on the data coming from the analysis of the WT1 promoter sequence and the data emerging form analysis of genes interactions in renal development and hematopoiesis. This work discusses interplay between series of transcription factors that were shown to contribute to WT1 upregulation in healthy tissues. Moreover, the mutation and methylation changes are discussed that may have potential influence on the upregulation of WT1 in common neoplasia.

7.1 Expression of WT1 and its regulators in human carcinomas

WT1 and PAX2, PAX8 genes, but not GATA1, are commonly expressed in human carcinoma cell lines and in tissue samples coming from patients. In both groups of samples the measurable expression levels seem to be more similar to those observed in acute myeloid leukemia than to those of healthy bone marrow (Figures 6.1-6.3). However, there doesn't seem to exist a direct qualitative or quantitative correlation between the expression of WT1 and its regulators in the carcinoma cell lines (6.4-6.5). Different picture emerges in the tissue samples where the expression levels of the PAX genes and WT1 gene seem to form a characteristic pattern. The low expression of WT1 seems to be independent of PAX genes upregulation whereas high expression of WT1 seems to correlate with upregulation of PAX gene mRNA expression above the certain threshold level. Those thresholds are 0,01 ratio for the WT1 gene and 0,0001 ratio for the both PAX genes. The expression of the studied genes above such ratios would be referred later in the work as the upregulation. The additional data supporting those hypothetical activation thresholds comes from acute myeloid leukemia where WT1 is expressed over such ratio in 82% of cases (Siehl J. personal communication). The control - 30 normal

bone marrow samples also expressed WT1 under this ratio. Therefore it seems to be also a clear

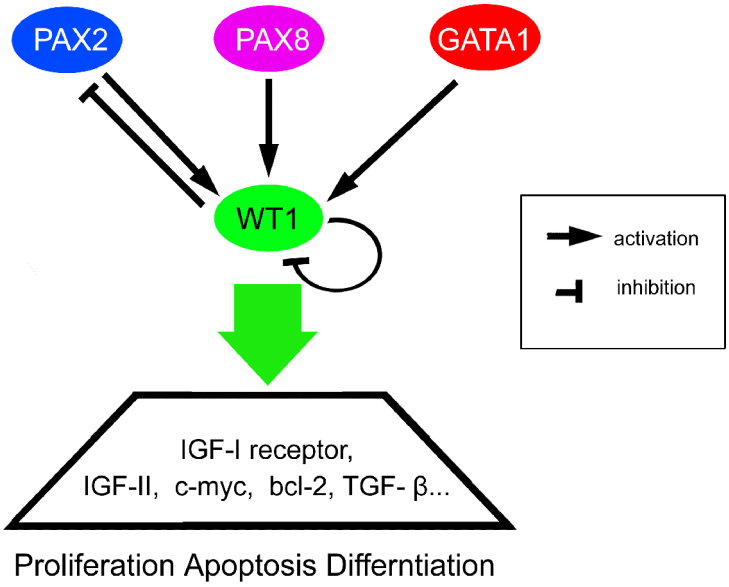

Figure 7.1. Model of WT1 expression regulation. WT1 might be activated by different factors (PAX2, PAX8, GATA1, Pea3, Sp1 and other). WT1 influences expression of different downstream targets, which have influence on cell proliferation, apoptosis and differentiation. The expression of WT1 is regulated by a feedback loop – WT1 is a negative regulator of its own expression and negative regulator of PAX2. PAX2 also interacts in vivo with WT to form transcriptionally active complexes.

cut-off ratio for distinction between pathological (AML) and normal WT1 expression. The main question that arises is if such WT1 mRNA expression leads to the synthesis of protein? The only comparable data comes from the work by Oji Y. et al. from 2002 on expression of WT1 in human breast cancers. In this work the expression levels of mRNA and WT1 protein were measured in K562 cell line and in a group of breast cancer cell lines and tissue samples. The comparison with our data shows that expression of the WT1 mRNA at the ratios of 0,0001, ratio over 100 fold lover than the proposed upregulation threshold, is connected with expression of functional protein in over 90% of cases in the group of breast cancer tissue samples analyzed in that work. So far there is no comparable data for PAX2 and PAX8 genes. The physiologic expression was studied only in few tissues and the levels of mRNA expression were not compared with protein expression.

If the observed activation thresholds are true, it may have several implications for future studies of the interplay between those three genes. First, the interactions between the genes should be analyzed in the cell lines which show upregulation of the genes above stated thresholds. If the expression of the genes does not reach the threshold there is a big chance that for some reason the interaction is disrupted and the study may not bring the true result. For example, if the activator of WT1 is present in the cells at very low levels and the WT1 is expressed at high levels at the same time, it may mean that the upregulation of WT1 is caused by other factor. In such

a cell line suppression of expression of activator would not lead to suppression of WT1 expression. Second, if the activator gene is expressed at high level, high above the activation threshold, even almost total suppression of its expression may not sufficiently block synthesis of the protein. In such a context RNAi of the activator would also not produce WT1 downregulation. Proposed activation levels reflect in vivo interaction of the studied genes. Therefore, it might be a good compass for cell line choice for future experiments. Third, the abundance of WT1 expression is much higher in the tissue samples than in the cell lines and it doesn't seem to have any qualitative and quantitative correlation with PAX genes. This underlines the fundamental difference in the data from *in vitro* and *in vivo* experiments. The WT1 may be more important for the *in vivo* growth of neoplastic cells than it is in the cell lines and PAX genes might be crucial for WT1 upregulation in tissues. The cell lines may accumulate different mutations or other changes that may compensate for the lack of expression of WT1 activators or even WT1. Fourth, WT1 expression is present in the cases, which do not express any of the known WT1 activating factors. It raises possibility that other factors may contribute. Such factors as SP-1 and NF-κB were shown to be unable to activate WT1 expression alone. However, they might theoretically interact with other general activators in the deregulated milieu of neoplastic cell or an epigenetic change may render WT1 sensitive for such activation. Other possibility comes from yet undiscovered factors that could positively regulate WT1 expression.

Summarizing, the "on-off" model of activation of WT1 emerges from the analysis of WT1, PAX2 and PAX8 coexpression in human carcinomas. The upregulation of PAX gene expression above arbitrary threshold leads to the upregulation of WT1. The model seems to explain well data coming from tissue

samples but doesn't function in the cell lines where picture is complicated, most likely, by the accumulated genetic changes.

7.2 RNA interference

The RNA interference is a mechanism in which dsRNA introduced into the cell interferes with a homologous mRNA expression by activating the dsRNA dependent RNase complex that degrades mRNA transcript in the cells. Method bases on phenomenon observed first in *C. elegans*, where transfection with dsRNA caused degradation of homologous mRNA (Fire A., et al 1998). Similar mechanisms of mRNA degradation after introduction of dsRNA of homologous sequence were shortly discovered in other animal and plant species (Hamilton A.J. and Baulcombe D.C., 1999, Hammond S.M. et al., 2000). The major step in introducing this method as a tool in a contemporary molecular biology was omitting interferon activation, which is normally caused when mammalian cells are transfected with dsRNA.
A group of German scientists lead by Tushl showed that reduction of dsRNA size down to 21 nucleotides allows avoiding the activation of interferon pathway (Elbashir S.M. et al., 2001). The shorts dsRNAs were termed siRNAs – short inhibitory RNAs. Transfection of the mammalian cells with siRNAs leads to downregulation of expression of targeted gene. The genes expressed at high levels can be downregulated to only a few percent of control expression whereas gene expressed at low levels can be downregulated to the levels below detection. The method shares similarity with antisense method of gene downregulation. The siRNAs and antisense oligonucleotides directed against same targets have similar effects. The main difference lies in the around 100 times lower doses of RNA that have to be used to achieve similar results and in the lack of interferon response in the case of siRNAs (Miyagishi M., et al., 2003). This is relatively new method of turning off the gene expression. The main advantage of this method is ability to switch of expression of genes that are necessary for organism development thus cannot be studied in classical "knock-out" fashion. WT1, PAX2, PAX8 and GATA1 are essential transcription factors and the RNAi seems to be an ideal method for analysis of the interactions between the expression of these genes.

7.2.1 Optimization of cells transfection efficiency

The RNA interference studies were undertaken to elucidate the relation between expression of WT1 and its regulators. The crucial element in RNAi experiments is the efficiency of transfection. There are few methods that have been used so far for efficiency analyses.

First one relies on introduction of a reporter gene (e.g. GFP) into the studied cell line. Efficiency of RNAi of that gene in the optimization experiments is thought to reflect the efficiency of the transfection. The potential drawbacks come from need to introduce foreign protein into the cells and the need for stably transfected cell lines. Moreover there is no possibility to control efficiency of transfection in each experiment – as the cells can be either transfected with control or experimental siRNA. The reporter gene may also exert some effects on cell livability and metabolism. In addition, the stable transfection of cells with reporter gene is not always possible.

Second method bases on RNAi of a housekeeping gene. It excludes the need to introduce a new gene but has another drawback – the efficiency can be measured only after the experiment. This means that there is no possibility of monitoring the efficiency during the experiments. It also needs a substantial amount of work and time – the quantitative PCR or Western Blots analyses must be done to answer simple question of transfection efficiency.

Additionally, in both methods the measured effects do not reflect the real efficiency of transfection but more the combined transfection rate and RNAi of the targeted genes – thus they are dependent on both processes.

The third method was chosen for the experiments presented here. It bases on staining of siRNA with fluorescent dye. Transfection efficiency can be analyzed shortly after the transfection on FACS scanner. This saves time and allows analysis of efficiency in each of the experiments and not only in optimization studies. There is no need for introduction of foreign genes or reporter proteins into the cell lines, and no need for isolation and analysis of gene/protein expression. The potential drawback is cost of fluorescent dye but this is more than paid off by reduction of other costs (qRT-PCR, Western blot, reporter constructs) and labor (protein, mRNA isolation/cDNA synthesis). The screening of transfection rate with FACS also simplifies procedure of optimization and allows rapid screening of transfection

efficiency in many samples in one experiment – which is impossible or time consuming in other methods.

The most of the cell lines used in this work in the RNAi experiments were not adherent. The nonadherent cells are harder to transfect with siRNAs than adherent cells with the standard method of siRNA transfection relying on transfer of siRNAs into the cells with liposomes. Actually, there was no data available on how to perform such experiments at the time that this study was undertaken. The incubation of cells on ice 10 minutes prior to transfection proved to be easiest way of improving the transfection efficiency. By this simple modification of standard protocol transfection efficiency was raised in some cell lines to virtually 100%. However, it seems that the transfection efficiency is strongly dependent on cell line. The observation was made that most of the cell lines either are easy or hard to transfect. If the transfection rate is high (over 50%) for a given cell line in initial experiments, usually it can be raised further up to virtually 100% by protocol modification. However, if the transfection rate is low in the initial experiments it will probably be hard to reach satisfactory transfection efficiency. It is reasonable to test multiple cell lines in the initial experiments and select these that are prone to transfection. The algorithm presented here shoes very simple way of analyzing and improving the transfection rate in nonadherent cells.

7.2.2 RNAi of the housekeeping genes

In control experiments the two house keeping genes were targeted: GAPDH and PBGD. GAPDH is a recommended standard target for RNAi control experiments and PBGD is a standard gene used as a reference gene in qRT-PCR. siRNAs against both genes were synthesized in vitro and used at the same concentrations in the studied cells. The commercially available GAPDH siRNA exhibited rather small activity (reduction of gene expression to 60% of controls) when compared to the self-designed PBGD siRNAs (reduction of gene expression up to 20% of controls). The activity of the siRNAs against GAPDH was much lower in my experiments when compared to manufacturer results. The differences may come from usage of different qRT-PCR systems or from different cell susceptibility to studied siRNAs. On the other hand, the reduction of expression of PBGD that was achieved by the method used was satisfactory. There were only mRNA levels measured in the

experiments but the reduction of the mRNA expression by RNAi to 20% of control as sensed by qRT-PCR was usually enough to suppress relevantly expression of the targeted protein (Ambion web resources). It was also suggested that when qRT-PCR is used for assaying the gene activity the best results are achieved when siRNAs target mRNA at the sites upstream the amplicon. However, in my experiments the action of most active siRNAs was not connected with relative target position on mRNA sequence.

The PBGD was targeted at many sites by different siRNAs to assure that RNAi of that gene will not produce any unspecific effects, such as off-target gene inhibition. Only a minor growth and total mRNA synthesis inhibition was observed under all of the PBDG specific siRNAs thus the unspecific action is unlikely. In a similar fashion the other genes were targeted to assure that similar effects are achieved by all siRNAs. Moreover, the siRNAs that had no homology to any known sequences were used as controls to assure that there are no siRNA specific but sequence unspecific effects. Additionally the control was included in which the cells were transfected with Transfection Agent without the siRNA. There was almost no difference in cell growth and mRNA quantity harvested in the probes that were transfected with siRNAs against PBGD, GAPDH, Negative Control and Transfection Agent alone. Thus the low unspecific effects of the transfection procedure can be traced to Transfection Agent and are most probably explained by low-level toxicity.

7.2.3 RNA interference of WT1 and its regulators

The choice of cell lines for WT1 RNAi experiments was based on possible highest bias reduction that may come from the unknown factors. The cell lines were known to express WT1 and only one of the known regulators of WT1 – to exclude potential substitution of one activator for another. Moreover the levels of expression of WT1 and its regulator were chosen to lie a little above the activation thresholds stated in former experiments – so to reflect expression levels that were seen in tissue samples. The cell lines with either very low expression or very high were excluded from analysis based on assumption that very low or high expression of WT1 may be effect of interaction with other factors – inhibitors or activators. The next parameter that was analyzed was susceptibility of cells to transfection. The lines that transfected well with minor influence on growth were chosen to exclude unspecific

effects and assure proper transfection rate. In such cell environment the bias should be reduced and the effects of WT1 RNAi should be seen at best.

The reduction of WT1 expression was observed under the action of WT1 specific siRNAs. The substantial differences were measured between different cell lines and total WT1 expression reduction varied from 16% to 50%. However the expression ratio to housekeeping genes varied in much lesser extent – 46% to 84%. The visible differences show one key difference between housekeeping genes and growth regulators (in this case WT1). The expression of a housekeeping gene can be downregulated to a high extent – the effects of proliferation and survival of the cell can be substituted by other genes or are not so crucial for cell survival. When the key growth regulator is targeted such as WT1 gene – the downregulation under a given level may not be possible at all – the cells simply die or stop to divide and expression of the targeted gene comes from the surviving cells. This is well seen in the K562 experiments where the growth inhibition is proportional to the RNAi effect on the WT1 gene. The growth inhibition comes after the RNAi is seen on the mRNA level and last around 73 hours – which is the time that transiently transfected siRNAs are supposed to work. The effects on the housekeeping genes reflect in most of the cases the effect on the targeted growth regulator.

In all of the experiments direct targeting of WT1 caused stronger cellular effects than the targeting of the regulator. This may mean two things – that either activation of WT1 is not completely dependent on studied regulators and other transcription factors play a role, or that the RNAi is not complete and small amounts of protein are synthesized that activate WT1 expression. The strong correlation between expression of WT1 and its regulators was observed for high expression of WT1. Low expression of WT1 seemed independent of PAX2, PAX8 or GATA activation. There is a possibility that WT1 can be activated at some points of cell cycle by other factors – this could explain lack of apoptosis after the RNAi of WT1, but the reduction of proliferation rate. In such model the regulators (PAX genes, GATA) could fuel high expression of WT1 independent of cell cycle phase. However, at some time points in the cell cycle the expression could be activated at low levels by other factors. In such a model the direct downregulation of WT1 would have much stronger effect on cell state than the downregulation of WT1 regulators. This could possibly explain later proliferation inhibition or no influence on cell proliferation when

PAX genes were targeted in the experiments. This would also mean that expression of WT1 might be activated by other factors in the chosen cell lines.

The transiency of transfection is another matter that has to be discussed. The RNAi effects were shown to disappear in the transiently transfected lines after around 72 hours. This time was affected by median division time with the correlation of high cell division rate with shorter RNAi. Bringing this fact into the light may explain why the cells return to normal proliferation rate after around 72 hours. If the cells survive the time during which WT1 (or other key cell life regulator) is downregulated they should return to normal proliferation rate after the RNAi effect disappears. This was indeed seen in the proliferation experiments after the RNAi of WT1. The other time dependent factor is stability of protein – the more stable protein is the more likely the RNAi influence on cell proliferation will be lesser. There is no data available on stability and turnover of the studied proteins so the influence of this factor cannot be fully assessed in the given experiments.

The studied gene expression was shown as a ratio of absolute expression of studied gene to a chosen housekeeping gene. It is a standard approach to quantification of gene expression in cell samples. It is very convenient approach that allows comparison of gene expression in different samples. Problem appears when a gene in study affects the expression of used housekeeping gene. In case of this work such feedback existed between GATA1 and PBGD genes. The GATA1 is known regulator of the PBGD gene in some of the hematopoietic cell lines. Theoretically the downregulation of GATA1 should lead to downregulation of WT1 and PBGD gene. It was indeed shown in some of the studied cell lines. To overcome this problem a simple strategy was applied. First, the second housekeeping gene was included in some instances where there was possibility for such an interaction. Second as the control and RNAi samples had very similar cell number up to 48 hours post experiment the total expression of the genes per sample was measured. In this way it was shown that the downregulation of the studied genes was accompanied by the downregulation of the housekeeping genes. It is a relatively good method of assessing the RNAi effects as the cell number per sample doesn’t change in the first 24 or 48 hours after RNAi in most of the studied cell lines.

The other source of bias in RNAi experiments might come from different levels of expression of the same gene analyzed in different cell lines. The same gene expression may vary more than three fold between different cell lines. In high-expressing cells the successful downregulation of the regulator gene may not lead to downregulation of the target gene – as there is enough mRNA remains after RNAi to fuel protein synthesis. It also means that if the gene expression varies between the samples of the same cell line it may have substantial effect on results. This may explain that the downregulation of GATA1 caused the downregulation of PBGD expression only in the cell lines that expressed GATA1 at low levels and not in those that had high expression of GATA1. Similar phenomenon could possibly have influence when the studied gene is expressed at high levels. However to limit the bias that may arise in this way the cell lines were chosen that expressed the studied genes slightly above the proposed activation thresholds.

The other possible problem in RNAi experiments is off-target gene regulation. So far there is no consensus whether siRNA have common unspecific effects: in the first paper in the field interferon activation and off-target activation were excluded (Elbashir S.M et al., 2001). The later papers supported both selectivity (Chi J.T. et al., 2003) and off-target gene action (Jackson A.L. et al., 2003). The best way to exclude off-target gene regulation is analysis of multiple control genes and usage of multiple siRNAs for one target gene. With such an approach the off-target gene regulation might be excluded.

The targeting of WT1 gene lead to a relatively quick answer and growth inhibition. Targeting the regulator, such as GATA1 gene, also lead to the growth inhibition. The time that passed between the RNAi and the effect was much shorter in case of WT1 than in case of GATA1. This may well be explained by the interaction of the studied genes. In the model downregulation of WT1 causes direct proliferation inhibition and the downregulation of the GATA1 causes downregulation of WT1. This could well explain the time difference in cell answer to downregulation of the genes. In other experiments it was already show that the GATA1 and PAX2 genes are crucial for cell survival (Muratovska A. et al., 2003; Whyatt D. et al., 2000). However it was not shown in what way the downregulation of these genes leads do growth inhibition. From the data presented here it may be concluded that WT1 is the key and target molecule for those regulator genes.

When the work was started there were no studies of RNAi in use for research of interactions between the activator transcription factor and target gene that relied on the quantitative PCR. The interplay between the WT1 gene and its regulators studied in this work showed all the problems that RNAi might face in such experiments.

7.3 Sequence analysis of the promoter regions of WT1 gene and its regulators in AML patients

The promoter sequences of the WT1, GATA1, PAX2 and PAX8 genes were screened for mutations that could have influence on expression of these genes. There were only a few single base polymorphisms found in studied sequences – however they did not differ from the ones that were present in control group and from the PubMed data. Thus the promoters of studied genes are not likely site of mutation in AML so the pathological expression of those genes is very unlikely caused by mutation. Therefore the pathologic upregulation of WT1 is caused by other changes - pathologic activators expression or epigenetic changes. The changes apart from single base polymorphisms are doubtful to be present in studied promoter regions in AML since no changes were ever noted in the size of the PCR amplicons and extra bands were never found during the gel electrophoresis. It can be speculated that PCR reaction amplifies the DNA from the healthy cells and that the rearrangements are overlooked - however if such changes were present in promoter regions they would involve the first exons (which in part are amplified) and most likely made protein expression impossible.

There were some new variations found at the sites that are not associated with know WT1 regulators. The meaning of such variations has to be further elucidated.

The promoter sequences of GATA1, PAX2 and PAX8 are sites of extremely rare variations. PAX8 promoter sequence was identical in all of the samples. In the promoter of GATA 1 there are no known variations and in the studied group of samples there was only one sample with a single variation. PAX2 promoter

sequences had two single base variations. It is very probably that those promoters retain their function in AML.

7.4 Methylation analysis

Methylation changes of CpG residues are the most common epigenetic modulation of human genome. The pathologic hyper- and hypomethylation of promoter regions were shown to play an important role in the pathogenesis of many cancers. The pathologic methylation pattern was observed in promoter region of WT1 in human neoplasia. There is a vast array of methods basing on PCR that allow analysis of the methylation status of a given loci (for recent review see Dahl and Guldberg, 2003). For this analysis the novel quantitative methylation assay was developed that bases on quantitative Real Time PCR. The DNA is digested with methylation sensitive enzyme and the DNA quantity in the sample prior and post digestion are compared. This method has been developed independent to the method by the Singer-Sam (Singer-Sam et al., 1989). Both methods base on the quantitative PCR and digestion of DNA with methylation sensitive enzyme. The first difference is the way that the negative and positive controls are performed. The method of Singer-Sam based on introduction of an extra DNA plasmid in the sample, which serves as positive control of digestion. However there was no negative control in the reaction. The method presented here bases on including two control DNA samples – one fully methylated that serves as negative control, and other one, which is unmethylated and serves as the positive control. The methylation of the studied samples has always a value between those two values. This shows in one step the efficiency of digestion without need to further analyze PCR products. The second difference lies in the method of amplification. The method of Singer-Sam used semi-quantitative assay with radiolabeled DNA hybridization probes and was hazardous (due to toxic reagents), laborious and inaccurate. The method presented here takes advantage of Light Cycler real time PCR and allows rapid analysis of each methylated loci. The method can be also used for simple PCR analyzes of point mutation for virtually any loci. The other advantage is a simple adjustment for already established sequencing PCR. This is the main advantage when this assay is compared to bisulphate-modification based methods, which at the time are the standard for methylation analyses. The bisulfate method bases on chemical

modification of DNA during which methylated residues stay unchanged and unmethylated Cytosine are changed to Uracil. Thus for each analyzed loci DNA has to be modified and new primer set has to be designed and PCR conditions have to be established. The use of SYBR Green stain and the method presented here allows use of already developed primers for methylation analysis of a wanted DNA sequence. As the methylation pattern changes can be used as a molecular marker for disease (Siu L.L.P. et al., 2003) the method presented here could potentially be a simple tool for clinical monitoring of such changes.

The methylation of the WT1 enhancer was analyzed at the three sites. Two of the analyzed sites lie in the region of the minimal enhancer of WT1. In both of them the low methylation status of the site correlates with low expression of WT1, and high methylation status of these places correlates with high expression of WT1. The only know WT1 regulator that binds to the enhancer is GATA1 protein. However the analyzed sites are not GATA1 binding sites. The hypermethylation of the promoter regions is connected with gene inactivation. Data presented here suggest that hypermethylation of the enhancer could have opposite effect. One simple explanation could be interference of the methylation with function of some negative regulator of the WT1 enhancer. The methylation of these sites would block inhibitor binding and would enable for GATA1-dependent activation of WT1 gene. In the cases of low methylated enhancer the higher levels of GATA1 expression with low WT1 expression were observed. This can also be explained with the same hypothesis. As the sites are not methylated the negative regulator binds to these sites and blocks the enhancer action. It must be noted that the Hpa II position is also a site of a polymorphism in the group of AML patients. However the group with the Thymidine substitution of Guanidine has similar expression pattern to the low methylated group (data not shown). It is speculatory but it would seem that the same genetic element – in this case WT1 enhancer – could function as enhancer or as a silencer – depending on the transcription factors that are bound to it.

The work focused on explanation of expression of WT1 gene in human neoplasia. The WT1 expression in studied neoplasia was correlated with expression of known physiological regulators: GATA1, PAX2 and PAX8 genes. By means of RNA interference it was shown that inhibition of expression of the regulators leads to downregulation of WT1 expression. It was shown that the promoters of the studied

genes do not contain mutations thus are able to play a role in the regulation of expression of studied genes. Moreover the epigenetic changes that may be involved in pathological expression of WT1 were studied with newly designed quantitative methylation assay. It was shown that methylation at a specific loci correlates with high expression of WT1. It was suggested that a putative negative regulator of WT1 synthesis might exist that binds to the studied site.