SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Copy number alteration and overexpression of the gene WT1 in neoplastic diseases

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The PhD defense is scheduled for 11 am on September 2, 2015, at the lecture hall of Building "A", Department of Internal Medicine, Faculty of Medicine, University of Debrecen.

1. INTRODUCTION

1.1. The WT1 gene and protein, isoforms

The WT1 gene encodes a unique transcription factor, which can activate or repress expression of target genes depending on the chromosomal and cellular environment. The C-terminal part of the protein contains four zinc finger domains, while a region rich in proline and glutamine is found at the N-terminus.

The protein binds to TATA-less, GC-rich promoters. Genes regulating the embryonic development of the kidney, heart and gonads are found among specific targets of WT1.

Complexity of WT1's function is largely attributed to a high number of isoproteins. The 4 main variants were characterized in 1991: alternative splicing is possible at 2 sites, resulting in proteins that contain or lack a 17-amino-acid sequence encoded by exon 5, and a KTS sequence encoded by exon 9. More isoforms were found in later years, the overall number of possible variants stands at 36 at present. Data suggest that the difference between KTS-positive and KTS-negative isoforms is the major decisive factor from a functional point of view.

Insertion of the KTS sequence between zinc finger 3 and 4 makes the linker region more flexible, hindering the interaction of the 4th zinc finger and DNA. Accordingly, KTS-negative WT1 plays the primary role in direct DNA binding. On the other hand, evidence supports a posttranscriptional regulatory role of WT1 due to protein-RNA and protein-protein interactions. These functions are mainly attributed to the KTS-positive isoform.

1.2. The gene's role in embryonic development

WT1 regulates the development of multiple organs. In sexual differentiation, it activates the promoter of Sf1, contributing to the formation and survival of the bipotential gonad. Without Sf1 or KTS-negative WT1, a streak gonad develops. WT1 also triggers the expression of SRY, and indirectly Sox9 and MIS, playing an essential part in male-type differentiation involving the regression of the Müllerian duct.

Kidney development is also regulated by the gene product at several checkpoints: survival of uninduced mesenchyme, mesenchymal to epithelial transition, formation of

nephrons, and the differentiation and proper function of podocytes. Interestingly, WT1 seems to play the opposite role in the cardiovascular system. The gene is expressed in epicardium-derived progenitor cells, which only develop into fibroblasts or muscle cells after WT1 expression has been silenced. The observation is explained by a two-directional effect of WT1 on the WNT4 promoter: the protein can bind to a coactivator, CBP or p300 in the kidney, and a corepressor known as BASP1 in the epicardium.

1.3. Importance of WT1 in malignant diseases

WT1 acts as a classic tumor suppressor in the genitourinary system: the loss of both copies results in Wilms' tumor. Malignancies that feature WT1 as an oncogene are typically characterized by ectopic gene expression without mutation. Leukemias seem to be an exception as they often involve heterozygous mutations apart from the overexpression of wild-type WT1.

As explained above, WT1 may act as a mediator of mesenchymal to epithelial or epithelial to mesenchymal transition in different tissue types, which offers a possible explanation for its two-faced role as a tumor suppressor in the kidney and an oncogene in tumors of other origin.

Although WT1 is generally considered by doctors as the locus responsible for Wilms' tumor, the gene is thought to be the cause of only 20 percent of the cases. Likewise, only 2 percent of patients diagnosed with a sporadic Wilms' tumor carry a germline mutation. If, however, the tumor appears as part of a complex syndrome, defects involving the locus are more common.

WAGR syndrome consists of Wilms' tumor, aniridia, genitourinary anomalies and mental retardation, often accompanied by other symptoms. Deletion of the 11p13 chromosomal region including the loci WT1 and PAX6 has long been known as its cause.

Denys-Drash syndrome is a rare genetic disease featuring diffuse mesangial sclerosis leading to an early kidney failure, also associated with Wilms' tumor or pseudohermaphroditism. The syndrome is caused by point mutations in the WT1 gene, most often in exon 8 or 9, encoding zinc finger 2 and 3, respectively. Experimental evidence suggests an abnormal epithelial differentiation of podocytes as a central feature.

Frasier syndrome is characterized by male pseudohermaphroditism, streak gonads or dysgenetic testes, which may develop a gonadoblastoma. A progressive

glomerulonephropathy as seen above is not a rare finding in Frasier syndrome either, but the typical feature is slowly progressing FSGS. A mutation involving intron 9 of WT1 has been found in the background of the disease, and is held responsible for abnormal alternative splicing, causing a shift in the ratio of KTS-positive and -negative isoproteins.

The pathophysiological and prognostic role of WT1 expression has been studied in hematological malignancies, especially in acute leukemias and MDS. Only 2 years after the gene had been discovered, a report was published on its overexpression in AML as well as ALL, noting that it is expressed only during a blast crisis in cases of chronic leukemia. Later, WT1-expressing cases of AML were associated with an adverse prognosis.

Currently, WT1 expression is used by clinicians as a marker of minimal residual disease during remission, especially in AML patients. In ALL, the practical use of such a marker is surrounded by more controversy, while in lymphomas, very few publications are available on the gene's expression and its possible role. A few authors have suggested the need for more studies on the connection of WT1 and lymphoma, but the field has not received much scientific attention, and is still overshadowed by leukemia research.

2. OBJECTIVES

We worked with clinical research groups to study cases of malignant disease which were suspected to involve the gene WT1. I introduce our projects and specific aims below.

1. Finding the WT1 abnormality responsible for a unique clinical entity somewhat similar to Denys-Drash syndrome.

2. Confirming our suspicion that the classic portrayal of WT1-related anomalies is overly simplified. In other words, the molecular palette of WT1 defects is so diverse that in certain cases, the observed phenotype does not match any of the traditional diagnoses known from medical literature.

3. Studying WT1 expression in hematological diseases by qRT-PCR. Our main goal was to document the frequency of overexpression in barely-studied non-Hodgkin lymphomas.

4. Providing data on whether and how WT1-positivity affects prognosis in various non-Hodgkin lymphomas.

3. MATERIALS AND METHODS

3.1. Studied patients

One of our patients (Cs. Cs.) was diagnosed at the 1st Department of Internal Medicine at the University of Debrecen: his kidney biopsy showed FSGS. Cs. Cs. is a 30-year-old man. His family history suggested an inherited anomaly of the genitourinary system. The patient was born with ambiguous external genitals, and developed multiple neoplasms during his life (ALL, Wilms' tumor, cerebellar angioblastoma).

We used blood samples taken from the patient and close family members. We first looked for molecular signs of a suspected atypical Denys-Drash or Frasier syndrome, determining the sequence of WT1 exons and the relevant intronic region. Later, we analyzed the copy number of the gene and carried out immune staining for WT1.

Another group of our patients suffered in hematological malignancies: non-Hodgkin lymphoma or ALL. Between March 2007 and May 2012, we received blood samples from 53 patients, detected WT1 expression and used statistical methods to study the relationship of WT1-positivity and prognosis.

3.2. DNA extraction, PCR reaction to study WT1 exons

We used a GenElute Blood Genomic DNA Kit (by SIGMA) to extract DNA from blood samples, and performed traditional PCR reactions (40-45 cycles) to amplify individual WT1 exons along with adjacent intronic sequences.

Afterwards, we ran the PCR product on a 2-percent agarose gel, and cut out the appropriate band under UV light, using a razorblade. We used a Q-BIOgene – BIO 101 Systems Geneclean Kit to re-extract DNA for purification purposes, and sent the sample to Szeged Biological Research Center for sequencing.

3.3. Copy number analysis, immune staining

To determine the copy number of the WT1 gene, we used a qRT-PCR (Applied Biosystems 7500 Real Time PCR System) with assays developed for copy number

detection (TaqMan Copy Number Assay, Applied Biosystems). The assay RNase P served as an endogenous control.

Our aim was to study the two ends of WT1 (exon 1 and exon 10) separately. However, none of the assays ordered for exon 1 seemed reliable enough, so we decided to detect intron 1 instead. Apart from WT1, adjacent genes PAX6 and EIF3M were also studied to help determine the boundaries of seen deletions and duplications.

Immunohistochemical staining was performed using the rabbit polyclonal antibody WT1 C19 (Santa Cruz Biotechnology), which binds to the C-terminal epitope of the WT1 protein. The slides to be stained were made from formalin-fixed, paraffin-embedded (FFPE) samples of the patient's childhood Wilms' tumor and his kidney biopsy taken at the age of 30, showing FSGS.

3.4. RNA extraction, determining WT1 expression

We used PAXgene Blood RNA Kits to extract RNA. We measured RNA concentration by NanoDrop, and performed reverse transcription using High Capacity cDNA Reverse Transcription Kits (by Applied Biosystems) without delay.

We detected cDNA quantity by qRT-PCR with a pair of primers and a TaqMan probe specific to the gene. Other than WT1 we also needed a reference gene, and chose GAPDH from the literature. Rather than using the Ct value for further calculations, we specified a standardized numeric value indicating the quantity of transcribed WT1 mRNA per 10^4 GAPDH mRNA molecules. To determine a cut-off value over which samples were considered positive, we used a pool of samples from 35 healthy individuals.

We classified enrolled patients by diagnosis. Within the main groups, we established subgroups based on measured WT1 expression, and illustrated overall and disease-free survival on Kaplan-Meier curves. Observed differences seen between curves were confirmed by a logrank test.

4. RESULTS AND DISCUSSION

4.1. Finding the WT1 defect in the background of a complex cancer syndrome

The unique clinical entity found in the case of Cs. Cs. was first explained as either Denys-Drash syndrome involving a less serious, late-onset glomerulopathy or a Frasier syndrome causing Wilms' tumor instead of gonadoblastoma.

However, the sequence of WT1 exons and the intronic region held responsible for Frasier syndrome did not show anything out of the ordinary. In the next phase, we investigated if a copy number anomaly might be the cause of the disease. Results showed a complete deletion of the WT1 locus in the patient that did not involve the surrounding genes, while his sister showed an extra copy of the 5' region of WT1. We did not find any abnormalities in the parents or in the patient's brother, who had undergone surgery for embryonal carcinoma.

Two FFPE samples were stained: one taken from the patient's triphasic Wilms' tumor in 1978, and a newer one from his other kidney showing FSGS. We observed a normal pattern of WT1-positivity in the more recent sample, characteristic for tumor-free kidney tissue: only the podocytes expressed the gene. It is worth noting, however, that positivity was weak, and nuclear areas failed to be stained entirely.

A parallel evaluation of the patient's clinical phenotype and the genotype found in our study makes both the diagnosis of Denys-Drash and Frasier syndrome rather forced. Two possible options are classifying the disease as an unusual manifestation of the Denys-Drash/Frasier spectrum, or extending said spectrum to include deletion syndromes, we may define the patient's condition as a case related to WAGR syndrome. We believe the latter argument is supported by clinical and molecular evidence.

The found phenotype (Wilms' tumor and male pseudohermaphroditism not accompanied by aniridia, mental retardation or obesity) fits in well with accumulated data on the role of important genes located in the chromosomal region. It seems that WAGR and WAGRO syndromes known from the literature should not be considered as independent entities, but as manifestations of a wider deletion spectrum involving regions

11p13 and 11p14.1. Within this spectrum, our case represents a deletion of the single gene WT1 with the corresponding phenotype.

One of the most intriguing aspects of the case is a single patient with multiple neoplasms (ALL, Wilms' tumor, cerebellar angioblastoma). It is known that the treatment of childhood malignancies may account for secondary tumors later, but (apart from our own study) no case has been reported in which leukemia occurred first, followed by a Wilms' tumor. Certain cases of acute leukemia have been associated with structural WT1 defects, and a WAGR patient who developed AML was reported to have a mutation in the remaining copy of WT1. These data suggest that the malignancies observed during the patient's life difficult to explain by long-term effects of chemotherapy should rather be attributed to a cancer predisposition caused by the deletion of WT1.

In a nearly complete lack of literature data, it is not easy to answer the question whether the partial duplication of WT1 found in the patient's sister may account for her mild but persistent proteinuria. Unusual copy number alterations seen in the children of the studied family raise the possibility of unequal crossing overs occurring frequently in the gametogenesis of one of the parents. (The mother seems a more likely candidate, as the history of the father's side did not reveal any genitourinary diseases.)

4.2. WT1 expression in hematological malignancies

At present, detection of WT1 expression by qRT-PCR is mainly performed in cases of AML. While elevated expression can be found in myeloid and lymphoid cell lines alike, the genetic background is different, at least to a certain extent: e.g. a hypermethylation of the AWT1 promoter is known as a specific marker of myeloid neoplasia. In our own research, we quantified mRNA transcribed from the WT1 gene in the more rarely studied lymphoid malignancies: non-Hodgkin lymphoma and ALL.

Especially in lymphomas, literature data on WT1 expression and its clinical significance is surprisingly scarce. Authors of currently available publications stained the WT1 protein by immunohistochemical methods in lymphoma tissue and lymph nodes.

For the first time to our knowledge, we reported WT1-positive cases of mantle cell lymphoma: we detected the expression of the gene in 4 out of 8 cases. Overall ($p=0.0082$) and disease-free survival ($p=0.0177$) both showed significant differences in favor of WT1-negative patients. Despite the low number of patients, results are rather suggestive for a

possible prognostic role by WT1, which led us to emphasize the need for more, larger-scale studies to prove the connection.

One of the patients was enrolled in the study after his lymphoma (diagnosed 5 years earlier) had relapsed. An initially negative expression value (0.0026) was replaced by an unusually high level of expression (2.07) a year later, when his lymphoma started to progress into a myelodysplastic syndrome. Massive overexpression of WT1 seen in the above case is likely to be a feature of MDS rather than non-Hodgkin lymphoma. The observation underlines the importance of studying the gene's expression in lymphoma patients, especially in heavily pretreated cases involving a higher risk to develop into MDS.

Out of 9 peripheral T-cell lymphoma cases, 4 were found WT1-positive, but the expression did not seem to be associated with prognosis. 2 cases of MALT and 2 cases of Burkitt lymphoma were also studied; they were all found WT1-negative.

A smaller group of our cases (7 patients) were diagnosed not with lymphoma, but B-cell ALL. WT1-positivity was seen in 5 cases. Its effect on overall survival could not be assessed, as the 2 WT1-negative cases progressed quickly and lead to a very early death. In cases where a remission was achieved, however, we saw a difference between the survival of patients who stayed WT1-positive, and the ones who turned negative in response to therapy. (The observation was not significant at the statistical level, probably due to the low number of cases.) In ALL, our own results are in line with the conclusion of recent large-scale studies: while it is unclear whether WT1 expression alone has a significant impact on survival, changes in expression levels do have prognostic implications.

Summarizing the results, although our study is only a minor contribution to the literature of ALL (due to being small-scale), it presents a crucial new finding for researchers of non-Hodgkin lymphoma: WT1-positivity can be detected in more subclasses of the disease than previously thought, and in certain lymphoma types it may affect prognosis the same way it does in leukemias.

5. SUMMARY

WT1 encodes a unique transcription factor, which may activate or repress certain promoters depending on cellular cofactors, while one of the protein's isoforms also regulates its targets at the posttranscriptional level. The gene guides the development of the gonads as well as several other organs, and is involved in tumorigenesis as a tumor suppressor or as an oncogene in different malignancies.

One of our research projects involved finding the WT1 defect responsible for a complex clinical entity consisting of male pseudohermaphroditism, FSGS, Wilms tumor, ALL and cerebellar angioblastoma. After extracting DNA from the patient's blood sample, we amplified all WT1 exons by PCR, and had them sequenced. We also performed copy number analysis by qRT-PCR, and stained FFPE samples by an antibody against WT1.

Results showed a microdeletion restricted to the gene WT1 – accordingly, the patient's disease was classified as a special case of WAGR syndrome, which did not involve the PAX6 locus. A partial duplication of WT1 was detected in the patient's sister, which raises the possibility of an abnormality affecting meiotic crossing over in one of the parents.

In another study, we determined the frequency and prognostic significance of WT1 expression in non-Hodgkin lymphomas and acute lymphoid leukemia. We regularly took blood samples from each patient, and used qRT-PCR to assess WT1 mRNA levels and their changes over the observation period.

For the first time to our knowledge, we documented WT1-positive mantle cell lymphoma, by detecting RNA from circulating lymphoma cells. In the disease, WT1 expression was found to be associated with prognosis; a similar trend was apparent in ALL. WT1-positivity was present in peripheral T-cell lymphoma, but did not affect survival. Studied cases of MALT and Burkitt lymphoma all proved to be WT1-negative.

Our results suggest that apart from leukemias, WT1 might also become a prognostic marker in certain types of non-Hodgkin lymphomas.

6. PUBLICATIONS

6.1. Publications related to the thesis

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List of publications related to the dissertation

1.* Ujj, Z., **Buglyó, G.**, Udvardy, M., Vargha, G., Biró, S., Rejtő, L.: WT1 Overexpression Affecting Clinical Outcome in Non-Hodgkin Lymphomas and Adult Acute Lymphoblastic Leukemia. *Pathol. Oncol. Res. 20* (3), 565-570, 2014.
DOI: http://dx.doi.org/10.1007/s12253-013-9729-7
IF:1.806 (2013)

2. **Buglyó, G.**, Méhes, G., Vargha, G., Biró, S., Mátyus, J.: WT1 microdeletion and slowly progressing focal glomerulosclerosis in a patient with male pseudohermaphroditism, childhood leukemia, Wilms tumor and cerebellar angioblastoma. *Clin. Nephrol. 79* (5), 414-418, 2013.
DOI: http://dx.doi.org/10.5414/CN107276
IF:1.232

Total IF of journals (all publications): 3,038
Total IF of journals (publications related to the dissertation): 3,038

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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* Ujj, Z. and Buglyó, G. contributed equally to this work.

6.2. Lectures and posters related to the thesis

Buglyó G, Rejtő L, Mátyus J, Biró S, Ujj Z (2011) Clinical association of mutations and expression abnormalities of the gene WT1. 4th Molecular, Cell and Immune Biology Winter School, Galyatető (lecture)

Buglyó G, Biró S, Méhes G, Mátyus J (2011) WT1 mikrodeléció atípusos klinikai kép hátterében: pseudohermaphroditismus, Wilms tumor, leukemia, cerebellaris angioblastoma és FSGS. Magyar Nephrológiai Társaság XXVIII. Nagygyűlése, Eger (lecture)

Buglyó G, Rejtő L, Biró S, Ujj Z (2012) The role of WT1 as a prognostic marker in non-Hodgkin lymphomas and adult acute lymphoblastic leukemia. European Medical Students' Conference, Debrecen (lecture)

Buglyó G, Rejtő L, Biró S, Ujj Z (2013). WT1 as a marker of prognosis in non-Hodgkin lymphomas and adult acute lymphoblastic leukemia. 6th Molecular Cell and Immune Biology Winter Symposium, Galyatető (lecture)

Buglyó G, Rejtő L, Biró S, Ujj Z (2013) A WT1 gén expressziójának qRT-PCR-es meghatározása hematológiai kórképekben. Genetikai Műhelyek Magyarországon: XII. Minikonferencia, Szeged (lecture)

Buglyó G, Rejtő L, Biró S, Ujj Z (2014) A WT1 gén expressziója, mint prognosztikai faktor akut leukemiákban és non-Hodgkin lymphomákban. A DE Humángenetikai Tanszékének tudományos ülése, Debrecen (lecture)

6.3. Other lectures and posters

Beyer D, **Buglyó G**, Markovics A (2014) Malignus hematológiai kórképekre hajlamosító genetikai tényezők kvantitatív-PCR vizsgálata. A DE Humángenetikai Tanszékének tudományos ülése, Debrecen (poster)