Review

The WT1 Gene – Its Role in Tumourigenesis and Prospects for Immunotherapeutic Advances

INGRID LINDSTEDT, MICHELLE ALEXIUS LINDGREN, ERIKA ANDERSSON and WILHELM ENGSTRÖM

*Department of Biomedical Sciences and Veterinary Public Health,
Swedish University of Agricultural Sciences, Uppsala, Sweden*

Abstract. *The Wilms Tumour 1 (WT1) gene is a complex gene which was originally linked to suppression of cancer in kidneys. Studies of WT1-knockout mice confirmed the important role of WT1 in the pathogenesis of Wilms' tumour, a tumour which accounts for 95% of all childhood renal tumours. In such cases, the WT1 gene acts as a tumour-suppressor gene. Subsequent research has shown that the WT1 gene in many other cases acts as an oncogene, most prominently in leukaemia and lung cancer (even though these cancer forms can emerge as a result of many other aetiological factors). Since WT1 acts as an oncogene in many different organs, it is of great importance to evaluate how and when the WT1 gene and protein act. This information can then be used to develop immunotherapy to stabilize and treat different malignant diseases. Both phase I and phase II studies have been carried out on candidate vaccines with varying but overall promising results. The immune response does not always correlate with the clinical response, however, and the efficacy of the treatment is often limited. Further development is, therefore, needed to understand how vaccines can be improved, so that they, can hopefully fulfil a clinical role in the future.*

Wilms' tumour is a paediatric kidney tumour which accounts for 95% of all renal tumours in young children. Its peak incidence is between two and three years of age, even though it is by no means a common tumour. Since the first cases of Wilms' tumour were described (1), a large number of studies have been made on genetic alterations that may explain the occurrence its. Earlier results pointed at the deletion of a

Correspondence to: Wilhelm Engström, Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, PO Box 75007, Uppsala, Sweden. Tel: +46 18671193, Fax +46 18673532, e-mail: wilhelm.engstrom@slu.se

segment of chromosome 11p as a link to Wilms' tumourigenesis (2). This segment, originally regarded as a pure suppressor gene, was termed *WT1*. Subsequently, it was found that that it is not only in humans that tumours develop due to lack or presence of *WT1*. An example of this is nephroblastoma (Wilms' tumour) in pigs (3), which makes this gene of universal interest, *i.e.* not only in human medicine. As more data have been gathered, the *WT1* gene has been ascribed different roles in carcinogenesis. Although it seems clear that *WT1* really acts as a suppressor gene in Wilms' tumourigenesis, it has been shown to act as an oncogene in other malignancies, such as leukaemia, glioblastoma and lung carcinoma. This has stimulated researchers to find a way of developing an immunological treatment, *i.e.* a vaccine, that can prevent patients from developing different types of tumours, for example leukaemia and lung carcinoma. A successful outcome of this struggle would lead to a completely new dimension in cancer prevention.

Gene Structure and Functions

The *WT1* gene is a complex gene that consists of 10 exons located on human chromosome 11p13 (4). WT1 is characterized by at least 36 isoforms. All these isoforms have four Zn fingers on their C-terminus. Isoforms can occur, for example, by including or excluding the three amino acids lysine, threonine and serine (−/+KTS), which have their place between Zn fingers three and four (5). Other isoforms can originate from the use of an upstream and in-frame CTG start codon, an internal ATG start codon at the end of exon 1 and a residue in exon 6 subject to RNA editing (6). Some of the 36 isoforms have only been described in murine and human samples, whilst others can be found in all mammals. The only isoforms which can be found in all vertebrates are the KTS variants (6).

The WT1 protein has many different key roles in the normal development of the genitourinary system as well as in tumourigenesis. Mutations or deletion of the *WT1* gene

results in a large spectrum of different diseases, for example Wilms' tumour or Denys-Drash syndrome, some of which are discussed below. *WT1* encodes a nuclear protein in normal cells and tissues, whereas it acts mostly in the cytoplasm in WT1-expressing tumours (8)

WT1, with all its isoforms, has many functions. In some cases, it can act as a transcriptional activator, whilst it in others as a transcriptional repressor. An example of this is that WT1 has been found to drive the epithelial-to-mesenchymal transition (EMT) in the developing heart by activating a specific gene, (*Wnt4*) at the same time as it drives the mesenchymal-to-epithelial transition (MET) in the developing kidney by activating another specific gene. The interesting thing about this is that the *Wnt4* gene that is activated by WT1 in the kidney is simultaneously repressed by WT1 in the heart (9).

The first function attributed to WT1 was its effects on transcriptional regulation. Here, as well as in the EMT and MET, it appears as if its function is either as an activator or a repressor. It is also believed that the *WT1* gene is involved in RNA metabolism, since it is associated with splicing factors; it is especially +KTS isoforms that are active in this case. *WT1* may also be involved in mRNA transport or stability (10). *WT1* has also been linked to the translation process as the WT1 protein can shuttle between the nucleus and cytoplasm. This requires an interaction between WT1 and β-actin (8).

WT1 as a tumour-suppressor gene versus oncogene. The *WT1* gene is considered to mainly function as a tumour-suppressor gene in Wilms' tumour. A conceptual query then arises of whether mutations in the *WT1* gene or deletion of the *WT1* locus is an obligatory prerequisite for tumour development or not. However, this is apparently not the case. In adults, most tissues do not normally express WT1. If these tissues then for some reason express WT1, this can contribute to tumour development. This suggests that, in order for tumours to develop, mutations in the *WT1* gene are not needed in these tissues. It appears as if WT1 is normally expressed only in kidney podocytes in adults (6). Examples of cases where WT1 might play an oncogenic role are leukaemia, and colonic/rectal, breast, lung and brain cancer (11). The fact that WT1 appears to be a universal tumour antigen and that it normally should not be expressed in adult tissues (except for kidney podocytes) makes it an excellent immunotherapeutic target.

WT1 in Disease

Spermatogenesis. Mouse models have been used to investigate the *WT1* gene and its role in spermatogenesis. The *WT1* gene is specifically expressed in Sertoli cells, which are essential for spermatogenesis. Knockout mice were created and results showed that at 10 days *post-partum*,

the *WT1*-deficient mice showed meiotic arrest and spermatogonia which had failed to differentiate and accumulated in the seminiferous tubules. The basal membrane, however, was not affected (12).

Denys–Drash, WAG (Wilms tumour, Aniridia, Genitourinary anomalia and Retardation) and Frasier syndrome. Denys–Drash syndrome is caused by heterozygous mutations in the *WT1* gene. The syndrome is characterized by early-onset diffuse renal mesangial sclerosis, often combined with gonadal dysplasia leading to male pseudohermaphroditism and Wilms' tumour (13).

The WAGR syndrome is characterized by Wilms' tumour in combination with aniridia, genitourinary abnormalities and mental retardation. The children which develop this disease always have a chromosomal deletion at 11p13. This is the location of *WT1*, but it is also the position where other genes, such as *PAX6* are located. This may explain the other symptoms, apart from Wilms' tumour, which develop when *WT1* is deleted (14).

The Frasier syndrome is rare and characterized by progressive glomerulopathy, male pseudohermaphroditism and gonadal dysgenesis with increased risk of gonadoblastoma. It has been shown that it is caused by mutations in a motif located in intron 9 in the *WT1* gene (15).

Wilms' tumour. Wilms' tumour accounts for about 95% of all renal tumours in children. However it is extremely rare in adults. The tumour occurs both in sporadic form and in hereditary cases. Wilms' tumour arises from undifferentiated metanephric mesenchyme and can be caused by more than one developmental error and therefore there are many different subtypes of this tumour. The first gene to be identified as a cause of Wilms' tumour was the *WT1* gene (2), but other genes have been identified since (7).

Studies on mouse models have been carried out to investigate how the *WT1* gene plays a role in the development of Wilms' tumour. Several experiments have been performed where knockout mice were created. The first ones resulted in mice that totally lacked kidneys, gonads and adrenal glands. Thus, the role of WT1 in the development of Wilms' tumour remained unexplained (16).

Later, however, investigations with knockout mice have supported the idea that *WT1* acts as a tumour-suppressor gene in later stages of kidney development. It is not likely, though, that defects or loss of *WT1* are enough cause for a tumour to develop. When tumours lack functional WT1 protein, a mutation in a the *CTNNB1* gene is present (17). This indicates that this gene might be a survival factor for these cells. During the MET process in the early kidney, the WNT signalling pathway is activated, which leads to stabilization of β-catenin protein. This, together with factors of the T-cell factor family, activates WNT target genes. When
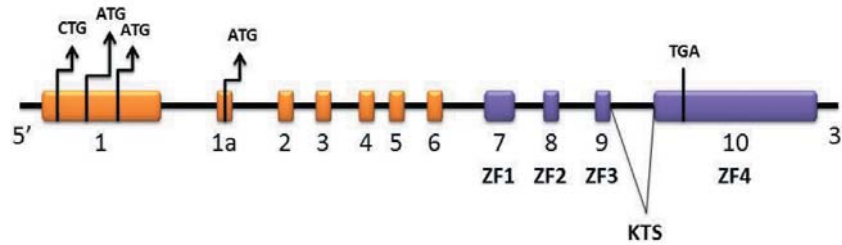
Figure 1. *An overview of the structure of the WT1 gene. Numbers 1-10 are the different exons. The four purple exons are the zinc fingers. Adapted from Royer-Pokora (7).*

the MET begins, cell division is reduced at the same time as WT1 expression is increased and WNT signalling pathway is down-regulated. For cells that lack functional WT1, however, an active WNT signalling pathway maintains proliferation of a cell population. One study has also shown that the *IGF2* gene might have the same function as *CTNNB1*, namely to keep the cells alive even without WT1 protein. This suggests that *CTNNB1* and *IGF2* act as oncogenes in this case (18).

Lung cancer. Some studies on lung cancer and its correlation with WT1 expression have shown that WT1 expression cannot be detected in normal lung tissue. However WT1 is expressed in high rate in lung cancer cells. Mostly it is 'normal' *WT1* genes that are expressed. Mutations and different isoforms are very rare (11). It has also been found that there is a correlation between prognosis and the expression of WT1. Studies have shown that high level of IgG antibody expression indicates a poor prognosis (19).

Leukemia. The WT1 gene is, except in Wilms' tumourigenesis, nowadays generally considered as an oncogene. That has also been the case for leukaemia. In normal peripheral blood or bone marrow, the levels of WT1 should be either very low or completely undetectable. Older studies have showed that in all acute lymphoid leukaemia and acute myeloid leukaemia (AML), *WT1* mRNA is highly detectable. It has also been observed that the expression of WT1 increases as the disease progresses in case of chronic myelogenous leukaemia (20) and myelodysplastic syndrome (MDS) (21). The level of WT1 has also been considered to be of potential use for prognostic determination (21). More recent studies, however, have shown that in most samples from patients suffering from acute T-cell leukaemia, the cells do not contain abnormal amounts of WT1 protein, even though *WT1* mRNA is highly detectable. These studies suggest that the role of WT1 is far more complex than just as tumour suppressor or oncogene. It has been shown that the –KTS isoform of the *WT1* gene promotes CD95-mediated
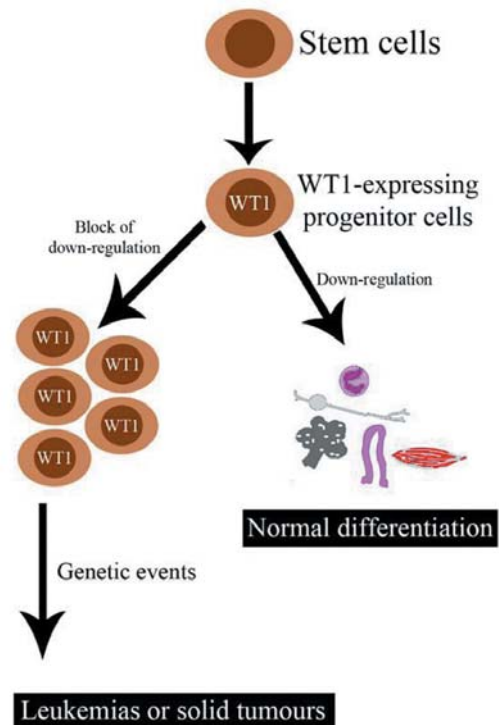

Figure 2. *An overview of WT1 tumourigenesis in leukaemia and non-kidney solid tumours. Adapted from Sugiyama (4).*

cell death in acute T-cell leukaemia. Silencing of *WT1* in these cells reduces CD95L expression, which leads to less apoptotic cell death (22). This would indicate that WT1 in this case has a tumour-suppressing rather than an oncogenic role. However, it has also been shown that many cancer cells express high amounts of CD95 and patients with cancer often have high levels of CD95L. These facts indicate that CD95 might have a growth-promoting role during tumourigenesis (23). This would then again argue against *WT1* as a tumour-suppressor gene in leukaemia and rather point to its being as an oncogene (Figure 2).

Vaccine Development

Early research. During recent years there has been more and more evidence that WT1 protein maybe a functional tumour antigen which could be used for development of a new kind of cancer vaccine. So far, vaccine-induced immunological responses have been detected (24). Research on this topic was aided by *in vivo* mouse models and human *in vitro* systems. It was then shown that immunization with either WT1 peptide or WT1 cDNA, induced WT1-specific CTLs in the mice. After immunization, the animals were capable of rejecting WT1-expressing tumour cells. The CTLs which were induced ignored normal tissues, which only expressed WT1 at normal, physiological levels. The studies on human *in vitro* systems were also positive and promising. It was shown that WT1-specific CTLs were induced, which were able to specifically lyse endogenous WT1-expressing tumour cells (25, 26).

Phase I clinical trials. After achieving sufficient pre-clinical results, research proceeded to phase I clinical studies in humans. One of the earliest studies was carried-out on two patients with MDS who were treated with WT1 peptide-based immunotherapy. It was shown that the number of granulocytes and lymphocytes were reduced after vaccination with WT1. The frequency of WT1-specific CTLs were increased and the number of leukaemia blast cells were reduced. All this occurred after just one single dose. The negative effects of the vaccination detected were severe leukopenia together with a local inflammatory response at the injection site (25, 26). However, it was unlikely that the leukopenia was the result of any damage to normal haematopoesis, since earlier studies had shown that there was no negative effect on normal organs, including the bone marrow (27).

Some subsequent phase I studies evaluated the skin toxicity of WT1 peptide vaccine, toxicity being graded on a scale from 1 to 4. One trial showed that the adverse effects in terms of skin toxicity were acceptable. Out of 10 patients, who altogether received 114 vaccinations, no grade 3 or 4 reactions were detected (28). Another clinical trial was performed on patients suffering from inoperable pancreatic or biliary tract cancer. Gemcitabine (chemotherapy) was combined with WT1 peptide vaccine mixed with incomplete Freund's adjuvant. The results of this study were that the number of CD14$^+$ monocytes and CD11$^+$ dendritic cells increased during the treatment, which can support the basis for this kind of treatment (29). When this study was finished, however, some patients still chose to continue the treatment. The primary goal was then to examine the adverse effects in terms of skin toxicity. Two out of the 25 patients developed severe (grade 3 or 4) and prolonged local adverse effects. This could have many different explanations, such as the fact that the injections were made intracutaneously and not intramuscularly. Such an effect could also depend on the concomitant usage of gemcitabine, since this increases the levels of dendritic cells and monocytes. The exact reason for the severe skin toxicity was not elucidated. However, this trial showed that it is very important that patients are adequately informed about potential side-effects and that they are frequently observed throughout the treatment (29).

Phase II clinical trials. Since the research in early trials and phase I studies generally generated positive results, phase II trials were initiated to further evaluate the technique with WT1 peptide vaccination. One phase II clinical trial was carried-out on patients suffering from AML. The aim was to evaluate the immunogenicity of WT1 peptide vaccination. The treatment with WT1 peptide was combined with treatment with granulocyte-macrophage colony-stimulating factor (GM-CSF), a cytokine which is used as an adjuvant and functions as a white blood cell growth factor. The treatment was well-tolerated and the results were overall promising. Many of the patients had stable disease after the treatment. Blast reduction and haematological improvement was seen in some patients. The results were not uniform in all patients however (30).

Another study on patients with AML was a combined phase I/II vaccination programme (31). The patients who participated had been treated with polychemotherapy, but were at high risk of relapse. They were then vaccinated with *WT1* mRNA-electroporated autologous dendritic cells. The results were promising, since it was shown that these dendritic cells were actually immunogenic and induced a measurable anti-leukaemic effect in these patients. The vaccination elicited both innate and adoptive immune response. Disease in some of these patients went into complete remission (and some into partial remission). Some of these patients, however, eventually experienced relapse. In conclusion, the findings of this trial support further development of this kind of technique to prevent relapse in AML (31).

The technique of using dendritic cells has also been tried on patients suffering from relapsed high-grade glioma. Repeated vaccination was combined with radiochemotherapy during several weeks. It was concluded that it was a feasible treatment without major toxicity, but the immunological response did not always correspond to the clinical response (32). Therefore a randomized clinical trial, which is prospective, double-blinded and placebo-controlled, was designed (33). According to the EU Clinical Trials Register, this trial is now ongoing and no results have yet been published (34).

Another phase II trial was initiated on patients with recurrent glioma. These patients had tumours that were resistant to standard therapy. Twenty-one patients were vaccinated every week for twelve weeks. The results showed

that the overall response rate was 9.5%. The rate for those who had complete or partial response, or stable disease was 57%. The adverse effects were limited to local erythema at the injection site (35).

Several different trials on gynaecological malignancies have been carried-out. These are diseases that are notoriously hard to treat once the diseases have become resistant to chemotherapy and radiology. The trials were therefore carried out on patients whose disease had developed these kinds of resistance. The results showed that the disease in some cases could be stabilized and did not progress for at least three months. In many cases, however, the disease was progressive. The adverse effects are mostly limited and largely tolerable (36, 37).

Other trials. Apart from the *in vitro* trials of early research, further *in vitro* studies (combined with *in vivo* studies) have been carried out. For example, one study investigated how eventual vaccines would affect podocytes, since these normally do express WT1, even in adults (38). Dysfunction of these cells could lead to complete renal failure and this is why it is very important that such a vaccine does not affect these cells in a negative way. Mouse cell lines and mice were used. In the *in vitro* test, T-cells showed cytotoxicity against the podocytes. *In vivo*, however, it seemed as if the podocytes were not affected at all. This was interpreted as being related to the anatomical localization of the podocytes. Since they are totally separated from blood vessels, they normally do not come into contact with the CTLs circulating in the blood. However, this would then mean that vaccinating patients who have glomerulonephritis would be of high risk, since CTLs could more easily infiltrate through the basement membrane and damage the podocytes (38).

Since many of the phase I and II clinical trials that have been made only have shown limited efficacy, some trials were carried-out to examine the reason for this immune evasion mechanism. One study focused on loss or mutation of *WT1* as a potential immune evasion mechanism causing limited efficacy. This was performed on patients suffering from AML who were in a phase II study. Blood and bone marrow samples were taken and examined but these showed no evidence of loss or mutation of *WT1* and therefore this was excluded as the immune evasion mechanism. Further studies in this field are necessary (39).

In another study, patients with myeloid malignancies were vaccinated with WT1 together with another peptide called PR1 in Montanide adjuvant and GM-CSF every two weeks for twelve weeks. An immunological response was shown in all patients after the first injection, but additional boosting did not increase vaccine-induced CD8^{+} T-cells any further. Before the sixth injection, the response was lost in all patients. These data may explain the lack of correlation between clinical responses and immune responses in many

clinical trials, since it may have a connection to the rapid loss of peptide-specific CD8^{+} T-cells when the peptides are delivered with Montanide adjuvant and GM-CSF (40).

Concluding Remarks

Since the *WT1* gene was discovered, its chromosomal localization, organisational features with respect to introns and exons, and its many different isoforms are well-known. However, it cannot be completely excluded that some hitherto undiscovered isoform(s) exist. Our knowledge about the function of the gene is far but complete. At least we know enough to be able to state that it is a developmentally conserved gene, which is of importance in many different species and in many different organs.

The *WT1* gene apparently has the capability to act both as a suppressor and a transforming gene that *per se* makes this particular gene very interesting. The key issue for this capacity is that WT1 is exclusively expressed in adult kidneys, irrespective of species (3, 41). This means that for tumours to develop in the kidneys, mutations in or deletions of the *WT1* gene are required. For tumours to develop in other organs, however, the expression of normal WT1 is enough to trigger tumour development. However, it should not be impossible that mutations in the *WT1* gene in other organs, apart from the kidneys, could exist and give rise to tumour development, even though these mutations are not actually required to trigger tumour development.

However, it might not be as simple as the *WT1* gene acts only as a suppressor gene or as a transforming gene. Many other different factors are needed, either to suppress tumours or to develop them. Some research results also contradict each other. Some suggest that the role of WT1 may also be tumour suppressing in the case of leukaemia, whereas others still claim that WT1 has an exclusively transforming role in this disease (42). The role of WT1 is highly complex, but since it has been shown that WT1 is not expressed in other adult organs, apart from the kidneys, it seems highly unlikely that *WT1* still should have a tumour-suppressing role in leukaemia. This remains an enigmatic issue and more research is required to completely understand the role of WT1 in different neoplastic diseases

What also makes the *WT1* gene highly interesting is the hope to use this knowledge as a foundation for the development of novel immunotherapeutic measures. This could result in disease prevention, as well as new therapeutic approaches to stop tumours from growing or to stabilize the neoplastic condition. This may be enough to give chemo- and radiotherapy a better chance to defeat the tumours that have already developed. In some cases, the immunotherapy in itself would hopefully be enough to combat the cancer.

It is also of great importance to investigate why a vaccine treatment is not always as effective as expected. This is to a

great extent linked to the low correlation between clinical response and immunological response. Once these issues have been resolved, it ought to be possible to proceed to phase III clinical trials.

Finally, it is important in studies of this kind to investigate exactly how and when the adverse effects arise. The different clinical trials had many different answers to this question, and therefore it is important to elucidate exactly what can be expected of a treatment of this kind.

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