The WT1-gene – its role in tumourigenesis and prospects for developing a vaccine

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The WT1-gene – dess roll i tumörutveckling och utsikter för att tillverka ett vaccin

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SAMMANFATTNING

WT1 genen är en komplex gen som ursprungligen är känd för att motverka canceruppkomst i njurarna. Studier på WT1 knockoutmöss har konfirmerat genens viktiga roll under sjukdomsutvecklingen av Wilms’ tumör, en tumörtyp som svarar för ungefär 95% av alla njurtumörer hos barn. I det fallet agerar genen som en tumörsuppressorgen. Senare forskning har dock visat att WT1 genen i många andra fall kan agera som onkogen, till exempel vid leukemi eller lungcancer (även om dessa cancerformer kan utvecklas som ett resultat av en lång rad andra etiologiska faktorer också). Eftersom WT1 verkar som onkogen i så många olika organ, är det av stor vikt att undersöka och utvärdera hur och när WT1 genen och dess protein utövar sin verkan. Denna information kan sedan användas för att utveckla immunoterapi (vacciner) för att stabilisera och behandla olika tumörer och cancerformer. Det har gjorts både fas I och fas II studier på detta område med varierande, men generellt lovande resultat. Immunsvaret korrelerar dock inte alltid med det kliniska svaret och effektiviteten av behandlingen är oftast låg. Ytterligare försök kommer därför att vara nödvändiga för att förstå hur vacciner och behandlingar kan förbättras för att sedan, förhoppningsvis, kunna användas kliniskt i framtiden.
ABSTRACT

The WT1 gene is a complex gene originally known to suppress cancer in kidneys. Studies of WT1 knockout mice have confirmed the important role of WT1 in the pathogenesis of Wilms’ tumour, a tumour which counts for 95% of all childhood renal tumours. In that case 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, e.g. in leukemia or lung cancer (even though these cancer forms can emerge as a result of many other aetiological factors). Since WT1 acts as an oncogene in so many different organs, it is of great importance to evaluate how and when the WT1 gene and protein acts. This information can then be used to develop immunotherapy (vaccines) to stabilize and treat different tumours and cancer forms. 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 very low. Additional trials are therefore needed to understand what can be improved in the vaccines and the treatment, so that they later, hopefully, can be used clinically in the future.
INTRODUCTION

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 case of Wilms’ tumour was discovered, a large number of studies have been made on genetic alterations that may explain the occurrence of this tumour. Results from the 1970:s pointed at the deletion of a segment of chromosome 11p as a link to Wilms tumourigenesis. This segment which originally was regarded as a pure suppressor gene was termed WT1. Subsequently it was found that that it’s not only in humans that tumours develop due to lack of/presence of WT1. An example of this is nephroblastoma (Wilms’ tumour) in pigs (Engström, et al., 2009) which makes this gene of universal interest, ie not only in human medicine. As more data has been gathered, the WT1 gene has been given different roles in carcinogeneis. Albeit it seems clear that WT1 really acts as a suppressorgene in Wilms tumourigenesis, it has been shown to act as an oncogene in other malignancies, such as leukemia, glioblastoma and lung carcinoma. This has stimulated researchers today to try to find a way of developing an immunological treatment, ie a vaccine, that can prevent patients from developing different tumours and cancer forms, for example leukemia and lung carcinoma. A successful outcome of this struggle would lead to a completely new dimension in cancer prevention.

MATERIALS AND METHODS

The articles used in this review were manly found via different on-line databases. Primarily Web of science, Pub med and Scopus were used. Many different search terms were used to find articles with different focus and approaches. For more general articles search terms as “WT1”, “Wilms’ tumour 1” and “nephroblastoma” were used. For more specific articles these words were combined with terms as for example “vaccine development”, “lung cancer” etc. Some articles were also given to me by my mentor.

LITERATURE REVIEW

Gene structure and functions

The WT1-gene is a complex gene that consists of ten exons and is located on human chromosome 11p13 (Sugiyama, 2010). The gene 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 KTS, lysine, threonine and serine (±KTS), which have their place between Zn-fingers three and four (Ozdemir et al., 2013). 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 (Hohenstein et al., 2006). Some of the 36 isoforms have only been described in mouse and human samples, whilst some can be found in all mammals. The only isoforms which can be found in all vertebrates are the ±KTS (Hohenstein et al., 2006).
The protein, which the WT1-gene encodes, 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. I will discuss some of these diseases below. WT1 encodes a nuclear protein in normal cells and tissues, whereas it acts mostly in the cytoplasm in WT1-expressing tumours (Dudnakova et al., 2010)

The WT1-gene, with all its isoforms, has many various functions. In some cases it can act as a transcriptional activator whilst it in other cases can act 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, 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 gene that is activated by WT1 in the kidney is repressed by WT1 in the heart at the same time (Assefi et al., 2011).

The first function of WT1 that was described 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 (Larsson et al., 1995). WT1 has also been linked to the translation process as the WT1 protein can shuttle between nucleus and cytoplasm. This requires an interaction between WT1 and β-actin (Dudnakova et al., 2010).

**Wt1 as a tumour suppressor gene versus oncogene**

The WT1-gene is considered to mainly function as a tumour suppressor gene in the case of Wilms’ tumour. Problems then arise when mutations in the WT1-gene appear or if the cells, which should be expressing WT1, whatever the reason, completely lack WT1. However, this is not always the case. In adults many tissues do normally not express WT1. If these tissues then for some reason express WT1, this can contribute to tumours development. This also means that, for tumours to develop, no mutations in the WT1-gene are needed in these tissues. It appears as if WT1 normally is only expressed in kidney podocytes in adults (Hohenstein et al., 2006). Examples of cases where WT1 might play an oncogenic role are colon/rectum
cancer, breast cancer, leukemia, lung cancer and brain cancer (Wang et al., 2013). The fact that WTI appears to be a universal tumour antigen and that it normally shouldn’t be expressed in adult tissues (except for kidney podocytes) makes it an excellent immunotherapeutic target. This will be discussed in more detail later on.

**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 ten days post partum, the WT1-deficient mice showed meiotic arrest and spermatogonia which has failed to differentiate and accumulate in the seminiferous tubules. The basal membrane, though, was not affected (Zheng et al., 2014).

**Denys Drash, WAGR and Frasier syndrome**

Denys Drash syndrome is a syndrome 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/or Wilms’ tumour (Patek et al., 2007).

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 (Caignec et al., 2007).

The Frasier syndrome is a rare syndrome which is 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 site in intron 9 in the WT1-gene (Gwin et al., 2008).

**Wilms’ tumour**

Wilms’ tumour is a tumour which accounts for about 95% of all renal tumours in children. The tumour is very 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, but other genes have been identified later (Royer-Pokora, 2012). However, in this article we will focus on the WT1-gene.

Studies on mouse models have been made to investigate in which way the WT1-gene plays a role in the development of Wilms’ tumour. Several trials have been made where knockout mice have been created. The first trials resulted in mice that totally lacked kidneys, gonads
and adrenal glands. Thus the role of WT1 in the development of Wilms’ tumour couldn’t be explained (Kreidberg et al., 1993).

Later on, however, trials with knockout mice have supported the thesis 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 is enough cause for a tumour to develop. When tumours lack functional WT1 protein, a mutation in a gene called CTNNB1 is present. 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 the MET begins, cell division will be reduced at the same time as WT1 expression is increased and Wnt signalling pathway is downregulated. For cells that lack functional WT1, however, an active Wnt signalling pathway maintains proliferation in a cell population. One study has also shown that the Igf2 gene might have the same function as CTNNB1, videlicet to keep the cells alive even without WT1 protein. This suggests that CTNNB1 and Igf2 act as oncogenes in this case (Hu et al., 2010).

**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 (Wang et al., 2013). 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 (Oji et al., 2009).

**Leukemia**

The WT1-gene is, except for Wilms’ tumourgenesis, generally nowadays considered as an oncogene. That has also been the case of leukemia. 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 leukemias and acute myeloid leukemias (AML) WT1 mRNA is highly detectable. It has also been seen that the expression of WT1 increases as the disease progresses in case of chronic myelogenous leukemia (Inoue et al., 1994) and myelodysplastic syndrome (MDS) (Cilloni et al., 2003). The levels of WT1 have also been considered to be of potential use for prognostic determination (Cilloni et al., 2003). More recent studies, however, have shown that most samples from patients suffering from acute T-cell leukemia, 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 cell death in acute T-cell leukemia. Silencing of WT1 in these cells reduces CD95L expression, which leads to less apoptotic cell death (Bourkoula et al., 2013). 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 cancer patients often have high levels of CD95L.
These facts indicate that CD95 might have a growth-promoting role during tumorigenesis (Chen et al., 2010). This would then again argue against WT1 as a tumour suppressor gene in leukemia and rather point it out as an oncogene once again.

**Fig 2. An overview of tumourigenesis in many cancer forms (not in kidneys). Adapted from Sugiyama, 2009.**

**Vaccine development**

**Early research**

During recent years there is more and more evidence for that WT1 protein could be a functional tumour antigen which can be used for development of a new kind of cancer vaccines. So far, vaccine induced immunological responses have been detected (van Driessche et al., 2012). Research on this topic was aided by in vivo mouse models and human in vitro systems. It could then be 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 could lyse endogenous WT1-expressing tumour cells specifically (Oka et al., 2006).
**Phase I clinical trials**

After achieving sufficient preclinical results, research could proceed to phase I clinical studies in live humans. One of the earliest studies was made on 2 patients with MDS, which 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 leukemic blast cells were reduced. All this occurred after just one single dose. The negative effects of the vaccination that could be detected were severe leukopenia together with a local inflammatory response at the injection site (Oka et al., 2003). However, it was unlikely that the leukopenia was the result of any damage to normal hematopoiesis, since earlier studies had shown that there was no negative effect to normal organs, including the bone marrow (Ohminami et al., 2000).

Some subsequent phase I studies have been made to evaluate the skin toxicity of WT1 peptide vaccine. The toxicity is then graded on a scale from 1 to 4. One trial showed that the adverse effects in terms of skin toxicity were acceptable. Out of ten patients, who altogether received 114 vaccinations, no grades 3 or 4 reactions were detected (Morita et al., 2006). Another clinical trial was made on patients suffering from pancreatic or biliary tract cancer, which were inoperable. 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 this kind of treatment (Soeda et al., 2009). 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 result could have many different explanations, as e.g. the fact that the injections were made intracutaneously and not intramuscular. It 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 eventual side effect and that the patients are frequently observed throughout the treatments (Soeda et al., 2010).

**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 made on patients suffering from AML. The aim was to evaluate the immunogenicity of Wilms’ tumour gene product 1 peptide vaccination. The treatment with WT1 peptide was combined with treatment with GM-CSF (Granulocyte-Macrophage Colony Stimulating Factor, 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 diseases after the treatment. Blast reduction and hematologic improvement was seen in some patients. The results were not uniform in all patients though (Keilholz et al., 2009).
Another study made on AML patients was a combined phase I/II vaccination programme. 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 (DC). The results were promising, since it was shown that these DC actually were immunogenic and induced a measurable antileukemic effect in these AML patients. The vaccination elicited both innate and adoptive immune response. Some of these patients’ diseases went into complete remission (and some into partial remission). Some of these patients, however, eventually relapsed. In conclusion, the findings of this trial support further development of this kind of technique to prevent relapse in AML (van Tendeloo et al., 2010).

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. The trial resulted in the conclusion that it was a feasible treatment without major toxicity, but the immunological response did not always correspond to the clinical response. Therefore a randomized clinical trial, which is prospective, double-blinded and placebo-controlled, was designed (Ardon et al., 2012). According to the EU Clinical Trials Register, this trial is now ongoing and no results have yet been published.

Another phase II trial was initiated on patients with recurrent glioma. These patients had tumors that were resistant to standard therapy. The patients were vaccinated every week during twelve weeks time. 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 (Izumoto et al., 2008).

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 made on people who had developed these kinds of resistances. 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 diseases were progressive. The adverse effects are mostly limited and largely tolerable (Miyatake et al., 2012) (Ohno et al., 2009).

**Other trials**

Apart from the in vitro trials that were made in early research, further in vitro studies (combined with in vivo studies) have been made later. For example, one study investigated how eventual vaccines would affect the podocytes, since these normally do express WT1, even in adults. Dysfunction of these cells could lead to complete renal failure and this is why it is very important that the vaccine does not affect these cells in a negative way. Mouse cell lines and mice were used. In the in vitro test, it turned out that T-cells showed cytotoxicity against the podocytes. In vivo, however, it seemed as if the podocytes weren’t affected at all. This was interpreted as if it had to do with the anatomical localization of the podocytes. Since they are totally separated from blood vessels, they do not come into contact with the CTLs...
which are circulating in the blood. This would then mean, though, that vaccinating patients with glomerulonephritis would be of a high risk, since CTLs then could more easily infiltrate through the basement membrane and damage the podocytes (Asai et al., 2014).

Since many of the phase I and II clinical trials that have been made only have shown limited efficacy, some trials have been made to examine the reason for this immune evasion mechanism. One study focused on loss, or mutation, of WT1 as a potential immune evasion mechanism. This was made on patients who were in a phase II study, suffering from AML. Blood- and bone marrow samples were taken and examined but these showed no evidence of loss or mutation of WT1 and therefore this can not be the immune evasion mechanism, which is the cause of the limited efficacy. Further studies on this field are going to be necessary (Busse et al., 2010).

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 time. An immunological response was shown in all patients after the first injection, but additional boosting didn’t increase the vaccine-induced CD8+ T-cells any further. Before the 6th 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 (Rezvani et al., 2011)

**DISCUSSION**

Since the WT1 gene was discovered its localization at the chromosome, its organisational features with respect to introns and exons and its many different isoforms are well known. However, it shouldn’t be completely excluded that some hitherto undiscovered isoform/s exist. We also know a lot, but far from everything, about the function of the gene. At least we know enough to be able to state that it is a universal gene, which is of importance in many different species and in many different organs.

It is possible that the WT1-gene can act both as a suppressor and a transforming gene that per se makes this particular gene so interesting. The key issue for this capacity is that WT1 is exclusively expressed in the kidneys in adults, irrespective of species under study. This means that, for tumours to develop in the kidneys, mutations or a complete lack of the WT1-gene is required. For tumours to develop in other organs, however, the expression of normal WT1 is enough to trigger tumour development. This should not be impossible, though, 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 really required to trigger tumour development.

However, it might not be as simple as that 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 also could be tumour suppressing in the case of leukemia, whereas others still claim that WT1 has an exclusively transforming role in this disease. I think that there is a completely logical explanation for the results that suggest that WT1 could be tumour suppressing. The role of WT1 is highly complex, but since it has been shown that WT1 is not expressed in other organs, apart from kidneys, in adults, it seems to me highly unlikely that WT1 still should have a tumour suppressing role in leukemia. This remains an enigmatic issue and more research is required to understand the complete role of WT1 in different neoplastic diseases.

What makes the WT1-gene of such high interest today is the hope to produce vaccines and/or immunotherapy. This could result in disease prevention as well as new therapeutic measures to stop tumours from growing or to stabilize the condition. This would maybe 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.

The way I see it, the most important thing right now is to investigate the lack of correlation between clinical response and immunological response. Maybe a great deal of the answer to that lies in the unanswered questions about exactly how and when WT1 acts as a transforming gene versus a tumour suppressor gene.

It should also be of great importance to investigate why the 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. Hopefully it will soon be possible to proceed to phase III clinical trials. Hopefully, a lot of the queries that are now, will be cleared up in connection to that.

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

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