Identification, Validation and Implementation of Blastemal Biomarkers in Wilms Tumour

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Identification, Validation and Implementation of Blastemal Biomarkers in Wilms Tumour

Daniel Sehic

DOCTORAL DISSERTATION
by due permission of the Faculty of Medicine, Lund University, Sweden.

To be defended on November 28th, 2014 at 13:00 in
Segerfalksalen, Biomedical Center, Sölvegatan 17, Lund, Sweden.

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Professor of Paediatric and Developmental Pathology
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London, United Kingdom
The aim of this thesis has been to establish biomarkers for the blastemal element in Wilms tumour (WT) – the most common paediatric kidney cancer. Blastema is, together with epithelium and stroma, one of the three common histological elements of WT. WTs dominated by blastema after preoperative chemotherapy are classified as high risk tumours.

According to the SIOP2001 protocol used in most European countries today, there is no recommendation of using molecular markers for WT risk stratification and this assessment is based on clinico-histological parameters alone. Patients with high risk tumours receive an extensive treatment, which may result in severe long term side effects. To secure an accurate estimation of the amount of blastemal elements a marker for detection of WT blastemal cells could be a useful tool, indirectly leading to a more precise risk estimation.

Literature studies and gene expression data were used to identify potential markers for WT blastema. The protein expressions for candidate markers were evaluated by immunofluorescence on WT cell lines and paraffin-embedded WT tissue sections. Tissue microarrays were constructed to improve the efficiency of this process and the most prominent marker was also validated by immunohistochemical protein staining, to prepare it for clinical use.

The two proteins found to have the most specific expression in blastemal cells were SIX1 and CITED1. These proteins are transcription factors expressed during kidney development and both were shown to be highly expressed in the blastemal element of WT (89% and 100%, of WT cases, respectively). SIX1 and CITED1 also displayed some expression in the epithelial (25% and 64%, respectively) and stromal (52% and 48%, respectively) elements. Since particularly CITED1 was highly expressed in epithelial components, SIX1 was selected for further clinical evaluation.

In summary, SIX1 appeared to be a useful marker for defining blastemal elements in WT. SIX1 does not replace a pathologist’s evaluation and should only be used as a tool to help give a more accurate estimation of the extent of the blastemal elements in WT.
Identification, Validation and Implementation of Blastemal Biomarkers in Wilms Tumour

Daniel Sehic

Department of Clinical Genetics
Faculty of Medicine

Lund University
2014
“Things that are important varies highly, until you have children.”
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This thesis is based on the following articles, referred to in the text by their Roman numerals.


III. **Sehic D, Albayati A, Sandstedt B, Gisselsson D.** A dual colour immunohistochemical staining for differentiating epithelial and blastemal elements in Wilms tumour. Manuscript.

ABBREVIATIONS

ADCA Renal cell carcinoma (Renal adenocarcinoma)
AMER1 APC membrane recruitment protein 1
AP Alkaline phosphatase
B7-H1 B7 homolog 1
BWS Beckwith-Wiedemann syndrome
CCSK Clear cell sarcoma of the kidney
CD56 CD56 molecule (NCAM)
CITED1 CREB-binding protein/p300-interacting transactivator with Asparagine/Glutamic acid-rich carboxyl-terminal domain
CMN Congenital mesoblastic nephroma
CREB cAMP response element-binding protein
CTNNB1 Catenin (cadherin-associated protein), beta 1
Cy3 Cyanine 3
DAB 3,3'-Diaminobenzidine
DAPI 4',6-diamidino-2-phenylindole
DDS Denys-Drash syndrome
EYA1 EYA transcriptional coactivator and phosphatase 1
FACS Fluorescence-Activated Cell Sorting
FITC Fluorescein isothiocyanate
H19 Imprinted maternally expressed transcript (non-protein coding)
HIER Heat induced epitope retrieval
HPR Horse radish peroxidase
IF Immunofluorescence
IGF2 Insulin-like growth factor 2
IgG Immunoglobulin G
IHC Immunohistochemical chromogenic
IRX3 Iroquois-class homeodomain protein IRX-3
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<tbody>
<tr>
<td>LIN28</td>
<td>LIN-28 homolog</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>NWTS</td>
<td>National Wilms Tumor Study</td>
</tr>
<tr>
<td>PAX6</td>
<td>Paired box 6</td>
</tr>
<tr>
<td>PS</td>
<td>Perlman syndrome</td>
</tr>
<tr>
<td>RAT</td>
<td>Renal rhabdoid tumour</td>
</tr>
<tr>
<td>RED</td>
<td>Permanent red substrate-chromogen</td>
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<tr>
<td>SIOP</td>
<td>International Society of Paediatric Oncology</td>
</tr>
<tr>
<td>SIX1</td>
<td>Sine oculis homeobox homolog 1</td>
</tr>
<tr>
<td>SIX2</td>
<td>Sine oculis homeobox homolog 2</td>
</tr>
<tr>
<td>TGF-beta</td>
<td>Transforming growth factor, beta</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue microarray</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumour protein p53</td>
</tr>
<tr>
<td>UKCCSG</td>
<td>United Kingdom Children’s Cancer Study Group</td>
</tr>
<tr>
<td>WAGR</td>
<td>Wilms tumour-Aniridia-Genitourinary anomalies-mental Retardation syndrome</td>
</tr>
<tr>
<td>WT1</td>
<td>Wilms tumor 1</td>
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Wilms tumour (WT) or nephroblastoma is a kidney tumour that generally affects young children with their whole life ahead of them. Improved treatment protocols including combinations of chemotherapy have fortunately led to great advancements during the 20th century, which have resulted in a survival rate close to 90%. The treatment for each WT patient is determined based on the histological subtype of the tumour and the tumour stage, which describes the extent to which the disease has spread. Treatment protocols are different for the various WT subtypes and an accurate classification is, for this reason, important. One histological WT subtype classified as high-risk according to current European protocols is the blastemal predominant type. These tumours consist of at least 2/3 immature, so-called blastemal cells that share many features with the progenitor cells present in the embryonic kidney. Since this WT group is classified as a high-risk tumour they also receive one of the toughest treatments. Such treatment often produces many long-term side effects, so called late effects, which may affect these young patients for the rest of their lives. A biomarker for objective recognition of blastemal cells in WT could be helpful in clinical risk stratification. Such biomarker could not only secure a more accurate estimation of the blastemal component but also ensure a more uniform estimation among pathologists. This, in turn, could lead to a more correct evaluation of the tumour composition, thereby giving each patient a greater chance to receive optimal treatment. The aim of this thesis has been to discover molecular markers suitable for defining blastemal cells in WT, validate them and prepare them for clinical use.

Lund, October 2014
INTRODUCTION

History

Wilms tumour (WT), also called nephroblastoma, was first characterized by Dr Carl Max Wilhelm Wilms (1867-1918) in 1899. His diagnosis of WT was based on clinical and histological appearance, the latter typically being a triphasic renal tumour consisting of blastemal, epithelial and stromal elements.¹

In the early part of the 20th century, WT patients had a survival rate of less than 20%. The only treatment at this time was surgery.² Surgical procedures were gradually improved and when combined with radiotherapy, survival rates increased to about 47% during the 1940s.³ By the 1960s, chemotherapy (actinomycin D and vincristine) had been introduced, raising the survival rates further.⁴,⁵ However, survival was still different depending on the stage of the WT and chemotherapy treatment dosage. This called for controlled clinical trials to improve treatment.⁶-⁸

During the 1970s the survival rate for WT patients increased to around 60% and in the 1990s the survival rate rose to exceed 80% in most developed countries. The primary reason for this dramatic improvement in outcome was the increase of multi-institutional trials such as the National Wilms Tumor Study (NWTS), the International Society of Paediatric Oncology (SIOP) trials, and their treatment protocols.²,⁹ These protocols refined the role of surgery, chemotherapy and radiation when treating WT. The tumours were divided into favourable and unfavourable types according to stage and histology. The vast majority of WT patients received surgery. Patients with the most favourable type of tumours, such as non-anaplastic WT with stage I-II, were treated with conventional dactinomycin and
vincristine chemotherapy while unfavourable WT with stage III-IV and anaplastic type received triple-agent chemotherapy (dactinomycin, doxorubicin and vincristine) and radiotherapy. Recurrent WT, which had and still has a poor prognosis, was treated with more aggressive surgery, radiotherapy and chemotherapy.²

Today, more than a hundred years after Dr. Wilms first described WT, the survival rate has reached about 90%. The histological tumour appearance is still an important feature used to diagnose WTs, classify them into different subtypes and thereby divide the tumours into risk groups that define the treatment received.¹⁰,¹¹

Background

WT is the most common kidney tumour in childhood. It represents about 85% of all paediatric renal tumours and 7% of all paediatric neoplasms. Most WTs are unilateral and only 5% display bilateral lesions.¹² In Sweden about 10-15 children are affected by WT each year. This corresponds to around 1 in 10 000 born children.¹³ The overall survival rate for children with WT in developed countries is high, but still at least 10% succumb to their disease.¹⁰,¹¹ In developing countries the survival rate can be much lower than 50%; one of the major reasons for this is limited resources.¹⁴,¹⁵ The majority of the affected children are younger than 9 years, with a median age of 3-5 years.¹² Adult WTs exist, but are rare. Only 3% of the WT patients are over 16 years of age.¹⁶ Adult WTs as a group differ in various ways from their paediatric counterparts. For example, these often have more advanced disease and may show more complex acquired genetic aberrations.¹⁷ However, most of the teenagers with WT have similar outcome compared to paediatric patients.¹⁶

A minority of children that present with kidney tumours does not have WT. Differential diagnoses in these patients include both benign and malignant entities. The most important of these entities follow below.
Clear cell sarcoma of the kidney (CCSK) is a malignant tumour, making up 4% of all paediatric renal neoplasms. It was earlier classified as a WT subtype, but differ from these tumours histologically by not displaying associations with genitourinary abnormalities, hemihypertrophy, sporadic aniridia or renal differentiation anomalies. The median age of CCSK-patients is the same as for WT, 3-5 years. CCSK can by its morphology most times be distinguished from WT, but differentiation can be difficult if the WT contains large amount of stromal element.

Renal rhabdoid tumour (RAT) was also first described as a variant of WT. It is the most malignant kidney tumour and represents about 1-2% of the paediatric renal tumours. Most affected patients are in their first year of life.

Congenital mesoblastic nephroma (CMN) is the most common renal neoplasm in patients between 3-6 months of age. CMN was earlier classified together with WT but is now considered to be separate from it. CMNs are benign tumours with a survival rate of over 98%. They account for about 3% of paediatric renal tumours.

Renal cell carcinoma (also called renal adenocarcinoma; ADCA) makes up about 2-3% of all paediatric renal tumours. The incidence increases with age and is higher over the age of 5. Overall survival for renal cell carcinoma patients younger than 20 years is a little over 60%.

Other rare paediatric neoplasms found in the kidney are cystic nephroma, Ewing sarcoma of the kidney and rhabdomyosarcoma of the kidney. Cystic nephroma is a benign renal tumour, while the other two are highly malignant.

Syndromes associated with Wilms tumour

Most WTs (90%) are sporadic, while around 10% of patients suffer from tumour-predisposing syndromes.
One of these is the Wilms tumour - Aniridia - Genitourinary anomalies - mental Retardation syndrome (WAGR). WAGR is a genetic syndrome with a deletion or mutation in chromosome band 11p13.\textsuperscript{30,31} This region includes \textit{PAX6} and \textit{WT1}.\textsuperscript{32} Most patients display aniridia (absence of iris). Genitourinary anomalies are not always present and are more common in boys than girls. To get diagnosed with WAGR syndrome, aniridia together with at least another feature must be displayed. The risk of developing WT for these patients is about 50%.\textsuperscript{33}

Denys-Drash syndrome (DDS) is a rare disorder characterized by gonadal dysgenesis, nephropathy and WT.\textsuperscript{34} These patients have a 55% risk of developing WT, 16% being bilateral.\textsuperscript{35} More than 90% of these patients have an abnormality in the Wilms tumour suppressor gene (\textit{WTI}).\textsuperscript{34}

Patients with Beckwith-Wiedemann syndrome (BWS) are recognised at birth by macroglossia, omphalocele and macrosomia.\textsuperscript{36} WT appears in around 10% of BWS cases.\textsuperscript{34} This syndrome is associated with alterations in chromosome band 11p15.\textsuperscript{37} Mainly patients with telomeric defects in this region develop WT. This telomere region contains \textit{H19} and \textit{IGF2}.\textsuperscript{38,39}

Perlman syndrome (PS) is an uncommon genetic disorder. These patients have up to 40% risk of developing WT. PS is an autosomal recessive overgrowth syndrome characterised by foetal gigantism, visceromegaly, characteristic face, Wilms tumour and bilateral nephroblastomatosis.\textsuperscript{39}

Other syndromes that have been associated with WT are; Simpson-Golabi-Behmel syndrome,\textsuperscript{39} Sotos syndrome,\textsuperscript{39} Frasier syndrome\textsuperscript{40} and Bloom’s syndrome.\textsuperscript{41} In some of these syndromes WT is very rare. WT have also been connected with hemihypertrophy,\textsuperscript{42} neurofibromatosis,\textsuperscript{43} Fanconi anemia\textsuperscript{42} and isolated hemihyperplasia.\textsuperscript{39}

There is also a non-syndromic form of familial nephroblastoma. But this form is rare and represents only 1% of all WT cases.\textsuperscript{34}
Kidney embryogenesis and Wilms tumour origin

WT is believed to originate from nephrogenic rests, i.e. embryonic cells formed during kidney development that remain in an immature state in the human kidney after birth. Since WT histology shares features with the human embryonic kidney and its cell biology reflects aspects of normal kidney formation this hypothesis is highly probable. Only a minority of nephrogenic rests lead to formation of WT and only around 30% of WTs have been found to contain these rests. Nephrogenic rests are located in either perilobar or intralobar locations. Perilobar rests are associated with overgrowth and overgrowth syndromes, while intralobar rests are associated with deletions and mutations in the WT1 gene. The presence of multiple nephrogenic rests beyond 36 weeks of gestation is called nephroblastomatosis. It can occur in one or both kidneys and be difficult to distinguish from WT. Nephroblastomatosis patients have a small increased risk of developing WT.

Kidney formation starts with the intermediate mesoderm forming the Wolffian duct. The ureteric bud emerges from the Wolffian duct and invades the metanephric mesenchyme, also called the metanephric blastema. The ureteric bud then begins to branch, mesenchymal cells aggregate around the ureteric tips and stromal cells proliferate between these tips. The early aggregates of mesenchymal cells forming at the tips of the ureteric bud are referred to as condensing mesenchyme or cap mesenchyme. It consists of immature nephron precursor cells with an undifferentiated morphology and expresses embryonic transcription factors such as SIX1, SIX2 and CITED1. From these cells, pretubular aggregates are formed that undergo a mesenchymal-to-epithelial transition and transform into the epithelial renal vesicles, through comma shaped and s-shaped bodies which mature into tubular structures. Foetal kidneys reach their fully developed adult form and position by the 10th-12th gestational week. After this age the kidneys only grow in size by continuous proliferation and maturation of the metanephric blastema adjacent to the renal cortex. In normal post-natal kidney, all cells of the
metanephric blastema should either have undergone mesenchymal-to-
epithelial transformation or have matured into stromal or vascular cells of
various types.

The epithelial component in WT is histologically very similar to foetal
kidney epithelium. However, in WT it usually covers a less part of the total
tumour tissue than it does in normal kidney tissue. The opposite may be
said for the stromal element that, if present in WT it usually covers a great
part of the tumour which is not true in normal kidney tissue. The
blastemal component of WT is similar to the metanephric mesenchyme in
foetal kidney. Its contribution to WT tissue volume differs extensively
among patients. Epithelial and stromal cells are derived from the
metanephric blastema in normal kidney development. In a similar way, the
stromal and epithelial components in WT are thought to arise from the
tumour blastema.

Histopathology and risk classification

WT mainly consists of three different tissue elements: blastema, epithelium
and stroma. The blastemal element consists of highly proliferating cells.
Blastemal cells are usually densely aggregated with large nuclei and a high
nuclear/cytoplasmic ratio. Epithelial elements are typically identified as
forming irregular tubular structures in WT tissue. The stromal elements are
usually populated sparsely with cells showing spindle cell morphology.
Some cases also show diffuse or focal anaplastic features. These anaplastic
elements display irregular nuclei, highly variable cell size, and atypical
mitoses. Because many WTs are now treated by chemotherapy before
histological assessment, it is also common to find areas of haemorrhage,
fibrosis, and necrosis, commonly referred to as regressive changes.
Figure 1
An example of the three most common histological tissue elements in WT: blastema (b), epithelium (e) and stroma (s).

According to the SIOP2001 protocol used in most European countries today, WTs are classified into different subtypes based on the relative extent of these different tissue elements at histopathological examination of each tumour after chemotherapy. If a post-chemotherapy treated tumour consists of more than 2/3 viable blastemal, epithelial or stromal cells it is considered to be predominated by that certain cell component. If none of the tumour cell components exceeds 2/3 it is considered a mixed type and if the tumour displays extensive multifocal anaplastic features it is considered to be diffuse anaplastic type. WTs that are more than 90% necrotic are considered to be of totally necrotic type, while those with more than 2/3, but less than 90%, necrosis/regression are classified as regressive type. In some rare WTs, different heterologous tissue types like muscle, rudimentary cartilage, bone, fat tissue\textsuperscript{53,54} or neural tissue\textsuperscript{55} are observed,
but these have no impact on the overall classification. According to the SIOP treatment protocol, patients with blastemal and diffuse anaplastic tumour types have higher risk for recurrence and death than patients with other WT subtypes. Therefore these patients receive a more extensive treatment. The epithelial, stromal, mixed, regressive and focal anaplastic types are considered to be of intermediate risk. The totally necrotic type is the only WT that is considered as low risk. In summary, classification into risk groups is crucial for treatment and strongly based upon tumour histology. The risk groups are also directly connected to patient prognosis. It should be noted that blastemal type is only considered high risk in post-chemotherapy treated WT, for example in the SIOP protocol; in non-pre-chemotherapy treated WT it is considered as an intermediate risk group. According to the SIOP protocol no immunohistochemical markers are needed for the risk classification or diagnosis of WT.

Staging, treatment and late effects

The two most widely used WT treatment protocols are determined by SIOP and NWTS, respectively. The SIOP-protocol is used in Sweden, most of Europe and some countries outside the European continent, while NWTS is used mainly in North America. United Kingdom Children’s Cancer Study Group (UKCCSG) provides another treatment protocol used in the United Kingdom. The main difference between the NWTS and SIOP protocols is that the surgical removal of the tumour takes place prior to chemotherapy in the NWTS protocol while after chemotherapy in the SIOP protocol. There is no difference in patient survival rate between these protocols.

According to the commonly used SIOP2001 protocol the spread or extent of WT disease is divided into 5 stages. In stage 1, the tumour is confined to the kidney and surrounded by the renal capsule. In stage 2, the renal capsule is penetrated and/or the renal hilus and/or sinus are invaded. Blood and lymphatic vessels may be invaded as well but are completely resected in stage 2. In stage 3, the tumour has spread to nearby lymph nodes and/or the
adrenal gland. Tumours that rupture into the abdomen prior to or under surgery are also considered stage 3. In stage 4, distant metastases are present, most frequently to the lungs and sometimes the liver or lymph node metastasis outside the abdominal-pelvic region. All bilateral tumours are considered as stage 5.57

Late effects for treated WT patients are not unusual. More than half of WT survivors suffer from chronic health conditions and for a quarter of the survivors, these health conditions are severe. Some examples of chronic conditions are congestive heart failure, renal failure and hypertension. In particular, doxorubicin combined with radiotherapy increases congestive heart failure.58 Radiotherapy and some chemotherapy agents increase the risk of developing secondary cancer,59 especially at high doses of radiotherapy.60,61 Tissue growth in both bone and muscle within the radiation field are also disrupted resulting in poor development. Even though radiotherapy might cause infertility, chemotherapy treatment in general does not affect the future fertility of the patients.59 Cyclophosphamide however, used in high-risk patients, may have a negative effect on sperm count.62 Hereditary factors in some syndromes also increase the risk of secondary cancer. Socioeconomic aspects of WT survivors in comparison to non-tumour patients seem not to be effected. Some examples of socioeconomic factors are educational accomplishment,63 employment64,65 and mental health outcome.65,66

Acquired genetic anomalies in Wilms tumour

The most common acquired genetic change in WT is alterations in chromosome arm 11p, especially affecting 11p13, location of \textit{WT1}, and 11p15, location of \textit{IGF2}.67 Loss of heterozygosity in 11p has been shown to be higher in bilateral WT.34 Even though the WT1 protein is expressed in almost all WTs,68 only 10-15% displays \textit{WT1} mutations.34 Still the WT1 protein is a commonly used diagnostic marker for WT. WT1 regulates IGF2 expression by its promoters.34 IGF2 is a foetal growth factor and
insulin receptor binding protein. Mutations in CTNNB1 have been found in about 15% of WTs. About 80% of WTs containing WTI mutations also have CTNNB1 mutations. β-catenin (the protein product of CTNNB1) has been shown to be regulated by AMER1 (also known as WTX). AMER1 is a tumour suppressor gene located in the X chromosome. It is expressed during embryonic development and inactivated in up to 20-30% of WTs.

Anaplastic WTs often have a high expression (76%) of nuclear p53 and the TP53 gene is mutated in most of these cases. 16q deletions have also been associated with blastemal and anaplastic subtypes. Loss of heterozygosity of chromosome arms 1p and 16q, both common features of anaplastic WTs, have also been associated with unfavorable prognosis in low-risk tumours. In some studies 1p allelic loss, and more recently 1q gain have been shown to be markers for poorer prognosis. Also, B7-H1 has been suggested as an unfavourable marker, if expressed in favourable histology WT.

Other chromosome areas often affected by somatic imbalances in WT are 7q, 7p, 11q, and chromosomes 12, 17 and 18.

Current focus in Wilms tumour research

Most patients presenting with WT today have a good prognosis. Still, about 10% succumb to their disease and naturally one important aim is to find strategies to cure patients with relentless disease.

Another goal is to minimize late effects for WT patients by optimizing treatment protocols. Any changes in treatment must first be investigated thoroughly according to standard guidelines for clinical trials. Standardised biomarkers to assist in predicting the clinical course of patients presenting with WT could be of great assistance in this process.

According to the SIOP 2001 protocol no molecular markers are currently needed for diagnosis and histopathological risk assessment of WT – routine histological staining such as haematoxylin-eosin should suffice. Still, to
support interpretation of routine histology a wide number of immunohistochemical protein markers are in current clinical use, such as cytokeratins to highlight epithelial components, WT1 for diagnosis on needle biopsies etc. This indicates that histological interpretation is not always straightforward and highlights a need for standardised immunohistochemical protein markers, especially for sub-classification of the tumours. This could also enable more straightforward comparisons between WT within and between different hospitals, regions or countries. Hopefully, such markers could help to further standardise the risk assessment of WT.

The aim of this thesis was to find clinically useful WT markers through exploration of bioinformatic data followed by extensive validation. Since markers for blastemal elements in WT seemed most needed, the main goal was to discover markers that could detect and quantify these elements.
THE PRESENT STUDY

Aims

To find potential molecular biomarkers for the blastemal component of WT, in order to facilitate a more accurate evaluation of the amount of blastemal cells in WT.

To validate such potential molecular biomarkers extensively on WT cell lines and WT paraffin embedded patient material.

To adapt potential molecular biomarkers for clinical use through an optimised immunohistochemical protocol.

To evaluate how well orthotopic xenograft models of WT, based on cell lines, recapitulate WT biomarker patterns used in the clinic.

Materials and Methods

The overall strategy was first to identify genes overexpressed at the mRNA level in WTs compared to foetal kidneys, as the former typically have larger quantities of blastema than the latter. Genes overexpressed in WT were first validated by cross-dataset comparisons followed by a database search of protein function and possible expression in foetal kidney. Candidate proteins were assessed by immunofluorescence on WT cell lines and tumour tissue before translation into immunohistochemical protocols for clinical use.
Gene Expression Microarray Data

Publicly available microarray datasets of gene expression from WTs were used in Article I. Genes were selected through comparison between WT and foetal kidney and included for further studies if the ANOVA two group comparisons between WT and foetal kidney had p-values ≤0.05 in more than two datasets. All of the datasets were analysed using the Qlucore Omics Explorer (Qlucore AB, Lund, Sweden) software.

Cell Lines and Tissue Sections

Three WT cell lines were used in this study: CCG-99-11, WT-CLS1 and WiT49. CCG-99-11 is a recurrent/metastatic WT cell line that was obtained from Dr. Jonathan D. Licht, Division of Hematology/Oncology, Northwestern University, Feinberg School of Medicine, Chicago, USA. The WT-CLS1 cell line was commercially available from Cell Line Service, Eppelheim, Germany. WiT49 is an epithelial-anaplastic cell line that was obtained from Dr. Herman Yeger, Developmental & Stem Cell Biology, University of Toronto, Canada.

Paraffin embedded tissue from WT patients treated from 1991 to 2010, anonymized foetal kidney and rare paediatric renal tumours, such as CCSK, RAT, ADCA and CMN were received from the Department of Pathology, at Lund University and Regional Laboratories, Lund, Sweden and the Academic Medical Center, Amsterdam, the Netherlands. The studies were approved by the Ethics Committee for Southern Sweden (LU119/03).

Tissue Microarray

A tissue microarray (TMA) was constructed from paraffin embedded tissue from thirty primary WTs, two CCSKs, two RATs, two ADCAs and one CMN. Paraffin embedded relapse WT material from seven patients and three different foetal kidneys were also included. Cores of about 1mm in diameter from these different tissue samples were transferred into a new paraffin block. Multiple cores were chosen from most of the tissue samples.
Over 200 different cores were used for the TMA. The TMA paraffin blocks were cut and placed on glass slides.

**Detection of Protein Expression**

Commercially available antibodies exist as monoclonal or polyclonal. Monoclonal only targets one epitope on the antigen, while polyclonal targets multiple epitopes on the antigen. In this study several IgG type antibodies were used, both polyclonal and monoclonal. One disadvantage of polyclonal antibodies is that the antibody composition may differ between different batches, which will most likely not occur with a monoclonal. An advantage with polyclonal in comparison with monoclonal antibodies is that it can target the protein of interest even if a specific epitope is unavailable.

**In situ protein detection**

Paraffin embedded tissue sections were used for most of the protein detection methods in this thesis. A heat induced epitope retrieval (HIER) procedure was used to activate the antigen epitopes of the tissue making it possible for the antibody to bind to it. Before adding the antibodies, the tissue was often blocked with blocking solution to minimise unspecific epitope-antibody binding, thereby decreasing false positive detection or background signals.

**Immunofluorescence protein staining**

An indirect method of immunofluorescence (IF) with two antibodies was used to simultaneously identify two epitopes. In this case, the primary antibodies target selected human protein epitopes and the secondary antibodies target the primary antibodies. The secondary antibodies also carried fluorescent labels called fluorochromes. Two different fluorochromes were used: fluorescein isothiocyanate (FITC) and Cyanine3 (Cy3). A fluorescence microscope was used to identify the subcellular
location of the fluorochromes. Light with specific wavelengths excited the fluorochromes and light of different wavelengths were emitted. FITC has a peak excitation of 495 nm and a peak emission of 519 nm (green). Cy3 has an excitation peak of 550 nm and an emission peak of 570 nm (yellow). 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nucleic acids in the cell nucleus. DAPI has an excitation peak of 345 nm and an emission peak of 455 nm (blue).

**Immunohistochemical (chromogenic) protein staining**

An indirect method of immunohistochemical chromogenic (IHC) dual staining combining the chromogens DAB and RED was also used to simultaneously identify two different proteins. Here, a primary antibody binds to the selected human protein epitope. A secondary antibody is linked to an enzyme, horse radish peroxidase (HPR) or alkaline phosphatase (AP). The enzymes are activated with substrates which initiate chromogenic reactions. DAB (3,3’-diaminobenzidine) is oxidised in the presence of HPR and produces a brown insolvable polymer that sticks to the adjacent tissue. To prevent false positive DAB staining, H$_2$O$_2$ is used to block peroxidase naturally found in the tissue. AP together with a RED chromogen produces an insoluble red product. The nucleus was counterstained with haematoxylin. Haematoxylin is oxidized into haematin and in the presence of metal ions (Fe(III) or Al(III)) binds to phosphate groups of the nucleus.

**Fluorescence-Activated Cell Sorting**

To evaluate epitope expression in commonly used WT cell lines, Fluorescence-Activated Cell Sorting (FACS) was used. With FACS it is possible to analyse and/or sort cells by targeting cell surface proteins of interest with fluorochrome-conjugated antibodies. In a single cell solution, individual cells are isolated into drops which can be analysed and sorted according to their different antibody binding capacity.
Xenograft tumour models

Cells or tissues transplanted from one species into another are called xenografts or xenotransplants. In Article IV three different human WT cell lines were transplanted into the renal capsule of immunodeficient mice to create orthotopic WT xenograft models. These models were established in order to increase the understanding of WT growth mechanisms and preclinical drug testing.

Results

In Article I, a primary search for protein markers for the blastemal component in WT was made, based on three external microarray datasets containing gene expression signatures from a total of 82 WTs and 8 normal foetal kidneys. The WTs were compared with normal foetal kidney to test the hypothesis that at least some genes with higher expression in WT than in foetal kidney would reflect the relative abundance of blastemal cells in WT. Protein expression from 17 candidate genes was investigated further by immunofluorescence on WT cell lines and tissue sections. This selection was based on four criteria: genes highly expressed in WT in comparison with CCSK, genes related to kidney development and gene expressions correlating with SIX1 expression and gene expressions anticorrelating with IRX3 expression, Genes with a similar expression pattern as IRX3 were excluded, since Irx3 regulates tubular maturation during kidney development,\(^{79,80}\) and our aim was to identify genes associated with more primitive stages during foetal development. Most of the potential markers investigated proved to be expressed at equal or lower intensity in the blastemal cells than in epithelial or stromal cells. Two exceptions were SIX1 and SIX2, which displayed higher protein expression in WT blastemal cells than in epithelial or stromal cells. SIX1 was found to be more specific for blastemal cells than SIX2. SIX1 stained all but one and SIX2 stained half of the blastemal elements in our WT set.
In Article II, SIX1 and two other potential blastemal markers, CITED1 and CD56, were compared using a TMA containing WTs. Both CITED1 and CD56 had in the literature been shown to be potential blastemal markers.\textsuperscript{81,82} The data in Article II revealed CITED1 and SIX1 to be highly specific markers of the blastemal elements in WT (100\% and 89\% specificity respectively), while CD56 was expressed in blastema in only 74\% of cases, and showed frequent expression in epithelial and stromal elements.

In Article III, SIX1 was chosen for translation into clinical use. This was done by setting up a dual colour SIX1/pan-cytokeratin immunohistochemical staining protocol. By this protocol, SIX1 showed a staining pattern highly similar to the immunofluorescence staining in Article II. During the spring of 2014, SIX1 immunohistochemistry was re-validated with similar good results at the Department of Pathology, Skåne University and Regional Laboratories, Lund, Sweden and is in clinical use since June 2014.

Article IV evaluated the cellular phenotype of WT cell lines xenografted into immunodeficient mice. WiT49 displayed a triphasic WT-like histology with blastemal, epithelial and stromal features but did not metastasize. Both CCG-99-11 and WT-CLS1 showed metastatic spread. CCG-99-11 displayed a blastemal morphology while WT-CLS1 could not be strictly classified into any WT subtype. SIX1 and CD56 stained all WiT49 xenograft elements strongly. SIX1 showed a weak diffuse staining in both CCG-99-11 and WT-CLS xenografts, while CD56 displayed weak diffuse in CCG-99-11 and focal expression in WT-CLS. We concluded that these xenografts poorly reflect WT tissue.
Discussion

Summary of results

In the present studies, multiple potential biomarkers for WT blastemal elements were evaluated in both WT tissue samples and WT cell lines. A few biomarkers were also tested on orthotopic WT xenografts (based on WT cell lines).

The most promising markers for the blastemal elements proved to be SIX1 and CITED1. SIX2 and CD56 expression were also detected in blastemal tumour components but in a far less number of patients. SIX2 and CD56 were for this reason excluded as potential blastemal markers. SIX1 rarely stained stromal and epithelial elements, but did stain the vast majority of the blastemal elements in the WTs investigated. All blastemal elements and some epithelial elements displayed CITED1 staining. SIX1 expression was not detected in all blastemal elements but the expression in epithelial elements was less extensive than for CITED1. In this respect CITED1 proved to be less specific than SIX1 as a blastemal biomarker. This was the main reason why SIX1 was chosen as the primary blastemal marker for clinical implementation.

Difficulties in strategies and methods

The strategies used in this study to find potential blastemal biomarkers, might in some aspects prove less straightforward than first expected. The expression data used was not annotated according to WT subtype. Such annotation could have made it less difficult to discover potential blastemal biomarker candidates, since it would enable a comparison between blastemal predominant tumours and other histological subtypes. The mRNA gene expression data did not always correspond to the amount of protein being expressed, making it difficult to identify/extract potential proteins with high expression. The presence of multiple and different
amounts of histological elements in each WT, also made the prediction of protein expression for the blastemal element more difficult.

The TMA, introduced in Article II, proved to be timesaving by giving extensive protein expression data more quickly than when using conventional tumour tissue sections. A disadvantage of using the TMA was the size of the tissue cores, which sometimes did not represent all tissue components present in the tumour. Since WTs most often are triphasic and these different tissue elements not often are expressed evenly across the tissue, some elements disappeared when slicing through the tissue core samples. Multiple cores from each WT were acquired to prevent this.

Two in situ protein detection methods, IF and IHC, were used in this study. IF makes it possible to turn on and off the protein specific signals while observing the result in a microscope. This can be very useful when investigating co-localisation of multiple proteins since it is possible to view them one at the time or all at once. The IHC method is more stable, usually gives less background and can be viewed in a normal light microscope. Unlike IF, IHC signals do not fade quickly when they come in contact with light. In clinical work the IHC method would prove more useful. However when a comparison between multiple protein markers is needed, especially more than two, the IF method would be preferred. Therefore, IF was used as a primary method in order to study colocalization of potential blastemal biomarkers. However, for clinical implementation we developed an IHC protocol for SIX staining.

Both WT cell lines and tissue samples were used during the course of these experiments. Cell lines differ to a great extent from tissue samples, both in cell composition and genetic diversity. Cell lines often derive from one or a small number of cells and often display a poor similarity in comparison to the diverse WT tissue. However, cell lines also have advantages such as high reproducibility and the possibility to evaluate markers on a large quantity of similar tumour cell material. In the screening for blastemal markers the cell lines described in this study displayed results that often
varied when compared to the WT tissue result, making tissue samples the primary choice when investigating markers. In addition, the WT cell lines used during the course of this study were difficult to classify into the typical histological elements seen in WT patient samples. This strongly questions their representativeness of WT.

**Origin of Wilms tumour and principal problems in biomarker studies**

The origin of WT has not been fully understood, but it is believed that nephrogenic rests or similar embryonic cells are WT precursors.\(^8^3\)

Primary WTs most often contain more than one of the common triphasic elements. This suggests that a precursor cell could differentiate into other WT elements, similar to the differentiation of the metanephric mesenchyme in the foetal kidney during renal development.

If WT arises from a single progenitor cell that later differentiates into blastemal, epithelial and stromal elements, these cells would be closely related making it difficult finding a protein marker that could distinguish between them. They may thereby reflect a continuous spectrum of differentiation rather than distinct cell types. In fact in our study blastemal, epithelial and stromal cells most often displayed the same expression of the many investigated proteins.

**More or less aggressive blastemal elements**

Unlike in the SIOP protocol, the NWTS protocol does not classify blastemal predominant WTs as high risk. The major difference between these two protocols is routine chemotherapy treatment before removal of the tumour mass. SIOP standard uses pre-chemotherapy treatment and NWTS standard use chemotherapy treatment after tumour removal.\(^8^4\) The reason for this difference in risk classification can thus be explained by biological selection. Through chemotherapeutic treatment, drug resistant blastemal cells can be created through positive selection pressure, resulting
in the creation of an aggressive blastemal clone or clones forming new tumours leading to disease progression.

According to the SIOP-protocol a blastemal predominant WT is considered as high risk, while a non-blastemal predominant WT is considered as intermediate risk type. In this case both tumours have existing blastema but only one is considered to be of high risk.

Furthermore, not all chemotherapy treated blastemal predominant WTs result in tumour recurrence or mortality. Blastema predominance is not a flawless measurement for high risk prognosis and cannot alone be used as an accurate prognostic marker. This further underscores that the biology of the blastema differs from patient to patient.

Possibly, a future characterisation of differences in biology between blastemal cells in blastemal predominant WTs vs. non-blastemal types could help in dissecting features of blastema which predicts clinical behaviour. It can be added that none of the blastemal markers in this study could distinguish between more or less aggressive forms of blastemal elements.

**Present candidate biomarkers in Wilms tumour**

There are several marker often used for classifying WT. WT1 is the most well-known and is expressed in almost all WTs. Cytokeratin markers exist for many of the different epithelial cells in the body, but they are usually not specific for WT and kidney epithelium. Antibodies against CD56 often display a diffuse staining in WT, and TP53 is expressed in anaplastic tumours. Hence, no markers can accurately classify WT. This applies not only to WT markers but also to other biomarkers used against other tumour types. The markers could stain more than one tumour type or not stain all of the tumours of a specific type. This is well known and often solved by using several markers. In this study, SIX1, CITED1, CD56 and SIX2 displayed a more promising expression profile than other investigated
markers. SIX1 and CITED1 were superior in staining the blastemal elements of WT.

SIX1 encodes a homeobox protein similar to the Drosophila “sine oculis” gene product, is crucial for kidney development and is expressed in the cap mesenchyme during kidney embryogenesis.\(^{45}\) SIX1 is also a co-transcriptional factor and forms a complex together with EYA1.\(^{85}\) Mutations in EYA1 or SIX1 are known to cause branchio-oto-renal syndrome with malformations of the nose, ears and kidneys.\(^{86}\) About 40% of these patients have a mutation in the EYA1 gene,\(^{87}\) while SIX1 mutations are much less common. SIX1 is involved in the development of craniofacial tissues,\(^{88}\) sensory neurons,\(^{89,90}\) the thymus\(^{90-92}\) and the parathyroid.\(^{90-92}\) SIX1 is also overexpressed in a wide range of human cancers and is associated with disease progression in several neoplasms.\(^{93}\) In breast cancer SIX1 expression increases the risk of metastasis.\(^{94,95}\) In ovarian cancer it is correlated with poor survival.\(^{96}\) In rhabdomyosarcoma its expression increases with disease stage.\(^{97}\) In hepatocellular carcinoma SIX1 expression has been associated to venous infiltration, advanced stage and a decreased survival.\(^{98}\) Finally, in murine breast cancer it has been shown to promote epithelial to mesenchymal transition\(^{99}\) through a TGF-beta-dependent mechanism.\(^{95,100}\) SIX1 has so far not been shown to have any clear role in WT pathogenesis and its expression in blastema may well be a feature simply signifying a state of metanephric-like differentiation.

CITED1 is a member of the CREB-binding protein/p300-interacting transactivator with Asparagine/Glutamic acid-rich carboxyl-terminal domain (CITED) family proteins and is believed to be a transcriptional coactivator.\(^{101}\) CITED1 is expressed in progenitor cells during kidney development and remains active in WT.\(^{102,103}\) It is also expressed in liver development and hepatoblastoma, but undetectable in adult liver. Hepatoblastoma contains, as most WTs, an epithelial and embryonic/foetal mix.\(^{104}\) Downregulation of CITED1 has been shown to suppress intestinal tumour development\(^{105}\) and lymph node metastasis has been predicted with CITED1 expression as a positive marker.\(^{106}\) CITED1 was highly expressed
in most WT elements and could suggest the presence of embryonic features in all these different components.

CD56 is a neural cell adhesion protein and a member of the immunoglobulin superfamily. It is expressed in multiple tumours like neuroblastoma, rhabdomyosarcoma, brain tumours, small cell lung cancer, multiple myelomas and acute myeloid leukaemia.\textsuperscript{107} CD56 staining of the blastemal element in WT has displayed irregular results in various studies.\textsuperscript{108,109} The same irregular staining with positive or negative CD56 expression in different WTs has been seen in this study (Article II).

**The future of biomarkers and treatment targets in Wilms tumour**

SIX1 is by itself not a prognostic marker. It only helps to distinguish blastemal components from other WT components. This marker does not replace a pathologist’s evaluation and should only be used as a tool to help give a more accurate estimation of the extent of the blastemal component.

Established prognostic factors in WT are mostly non-molecular and include disease stage, histological subtype, tumour volume and patient age. One major improvement in treatment for WT patients would be to expand the range of these prognostic markers. Some examples of molecular markers to determine prognosis in WT are the loss of heterozygosity at chromosome 1p and 16q, \textit{TP53} mutations or B7-H1.\textsuperscript{78,110} High expression of \textit{Lin28} has also a prognostic value. It both prolongs renal development and promotes the forming of Wilms tumour. \textit{LIN28} expression is also associated to blastemal cells.\textsuperscript{111} The value of this protein as a blastemal marker still needs to be evaluated further. Combining multiple biomarkers could also prove to be a step forward in classifying WT risk groups and tumour prognosis.

It would be interesting to functionally investigate the role of SIX1 and CITED1 in the blastemal component of WT to give an indication if they could be possible therapeutic targets.
Conclusion

- SIX1 and CITED1 are useful markers to identify blastemal elements in WT.
- SIX1 is successfully introduced as a clinical marker for the blastemal elements in WT.
- The WT cell lines used in this study poorly reflect human WT tissue in regard to biomarker expression and morphology.


I denna studie har potentiella biomarkörer för det blastemala elementet i Wilms tumör undersöks. Detta för att hitta mer objektiva sätt att riskgruppera patienterna än patologisk klassificering. När denna studie påbörjades användes ännu ingen biomarkör för enbart den blastemala

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REFERENCES


20. Argani P, Perlman EJ, Breslow NE, Browning NG, Green DM, D'Angio GJ, Beckwith JB. Clear cell sarcoma of the kidney: a review of 351 cases from
the National Wilms Tumor Study Group Pathology Center. The American

official publication of the International Cancer Imaging Society 2007:7:41-
51.

22. Bolande RP, Brough AJ, Izant RJ, Jr. Congenital mesoblastic nephroma of
infancy. A report of eight cases and the relationship to Wilms' tumor. 

23. Howell CG, Othersen HB, Kiviat NE, Norkool P, Beckwith JB, D'Angio GJ. 
Therapy and outcome in 51 children with mesoblastic nephroma: a
report of the National Wilms' Tumor Study. Journal of pediatric surgery 

24. Bosquet M, Dominguez C, Balaguer J, Serrano A, Estornell F, Martinez-
Verduch M, Garcia-Ibarra F. Pediatric renal adenocarcinoma: a review of

25. Tsai HL, Chin TW, Chang JW, Liu CS, Wei CF. Renal cell carcinoma in
children and young adults. Journal of the Chinese Medical Association :
JCMA 2006:69(5):240-244.

sarcoma of the kidney: case series and literature review of an often
overlooked entity in the diagnosis of primary renal tumors. Urology 

Cortesi E. Clinical and pathological features of primary neuroectodermal


29. Mehrain R, Nabahati M. A case of rhabdomyosarcoma of kidney
mimicking nephroblastoma. Caspian journal of internal medicine 


65. Lahteenmaki PM, Sankila R, Pukkala E, Kyyronen P, Harila-Saari A. Scholastic achievement of children with lymphoma or Wilms tumor at the


