Orthotopic Wilms Tumor Xenografts Derived From Cell Lines Reflect Limited Aspects of Tumor Morphology and Clinical Characteristics

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INTRODUCTION

Wilms tumor (WT) is a pediatric tumor of the kidney, the treatment of which includes heavy chemotherapy. Affected children would likely benefit from more targeted therapies with limited side effects. Establishment of relevant orthotopic WT xenografts is important to better understand mechanisms of WT growth and for preclinical drug testing. Procedure. Here we established and characterized orthotopic xenografts from WT cell lines WiT49, CCG-99-11, and WT-CLS1 to ascertain in what aspects each of them recapitulated WT histology, immunophenotype, and metastatic spread. Results. WiT49 xenografts recapitulated near triphasic WTs with clear WT1 staining and anaplastic features, but with tumor restricted to the kidney. On the contrary both CCG-99-11 and WT-CLS1 xenografts conveyed metastatic disease. Conclusions. From the three tested cell lines, orthotopic WiT49 xenografts best reflect the triphasic pattern of classical WT. Pediatr Blood Cancer 2014;61:1949–1954. © 2014 Wiley Periodicals, Inc.

Background. Wilms tumor (WT) is a pediatric tumor of the kidney, affecting young children. Today most afflicted children survive, but with the drawback of sometimes severe treatment side effects. The need for WT models for evaluation of new therapeutic drugs is great, but only a handful of WT cell lines are at the disposal of researchers. Although, orthotopic WT xenograft models based on established WT cell lines have been designed there have as yet not been any in-depth reports addressing their representativity with respect to clinicopathological variables [1,2]. The present study addresses this issue by detailed characterization of orthotopic xenografts established by the three WT cell lines WiT49, CCG-99-11, and WT-CLS1. WiT49 is a cell line established from a WT lung metastasis. The primary tumor and lung metastasis expressed normal WT1 and had a WT recurrent P53 mutation [3]. WT cell line CCG-99-11 also derives from a WT lung metastasis [4], whereas WT-CLS1 was established from a primary epithelial WT. We here evaluate how well orthotopic xenografts in immunodeficient mice recapitulate WT histology and the natural course of untreated WT. We found that all three cell lines had anaplastic features and none of them was able to perfectly recapitulate the histopathology, immunophenotype, or natural course of the common subtypes of WT. CCG-99-11 had several features of blastemal type WT, while WT-CLS1 had some epithelial type features but was highly dissimilar from classic epithelial WT. WiT49, in comparison to WT-CLS1 and CCG-99-11, was the one best recapitulating the classic triphasic histology of WT, with compartments resembling blastema, epithelium, and stroma.

MATERIALS AND METHODS

Cell Culture

WiT49, WT-CLS1 (Cell Lines Service GmbH, Eppelheim, Germany) and CCG-99-11 cells were cultured in DMEM F:12 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

Animal Procedures

Female NSG mice, 4–6 weeks old (Charles River Laboratories, Wilmington, MA), were anaesthetized using 2–3% isofluorane inhalation and Temgesic was administered subcutaneously. An excision was made exposing the left kidney and 2 × 10^5 WT cells in 30 µl PBS were injected into the kidney. The mesentery membrane was closed with sutures and the skin was closed with surgical clips. Mice injected by either CCG-99-11 or WT-CLS1 were sacrificed immediately when they showed general symptoms of tumor growth. Mice injected with WiT49 cells showed no signs of tumor symptoms and these mice were sacrificed 141 days after tumor cell injection. The primary tumor, kidney, liver, and lungs were preserved in formalin for histopathological examination. If tumor infiltration was visible in other organs these were preserved as well.

Fluorescence Activated Cell Sorting (FACS)

Cultured WT cell lines were analyzed by FACS Aria II flow cytometry (Becton, Dickinson and Company, Franklin Lakes, NJ). The cells were harvested and resuspended in PBS with 2% fetal bovine serum and blocked with isotype control (Becton, Dickinson and Company, 555740). Antibodies and their catalogue numbers were as follows:

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used in the experiments were CD56 (345812, Becton, Dickinson and Company) and CD326 (324203 BioLegend, San Diego, CA), and their isotype controls (556655 and 556656 respectively, Becton, Dickinson and Company). Isotype controls were used to estimate the positive and negative cell populations for the two evaluated antibodies. Doubles were excluded from the analysis. The program FlowJo vX.0.6 (Tree Star, Inc., Stanford, CA) was used for data analysis.

**Immunohistochemistry, Immunofluorescence, and Mitosis Index**

The following antibodies (including dilutions and catalogue number) were used for immunohistochemistry and immunofluorescence: WT1, 1:50, M3561 (DAKO, Glostrup, Denmark); CKAE1/AE3, prediluted, 760–2595 (Ventana Medical Systems, Tucson, AZ); CD56/NCAM, 1:50, M7304 (DAKO); p53, 1:100, M7001 (DAKO); and SIX1, 1:1,000, HPA001893 (Atlas Antibodies, Uppsala, Sweden). Immunofluorescence procedures have been previously described [5]. Immunohistochemistry was performed according to standard methods.

**RESULTS**

**Immunophenotype of WT Cells by FACS**

In an initial experiment to differentiate surface markers between the three WT cell lines WiT49, CCG-99-11, and WT-CLS1, FACS analyses were performed on in vitro cultured cells. Cells were sorted by the cell adhesion molecules EpCAM (epithelial), and immature/neural CD56-NCAM. WiT49 cells showed high (68.3%) EpCAM, and high CD56-NCAM (93.9%) expression (Fig. 1A), likely revealing an inherent potential to form epithelial structures. Neither CCG-99-11 nor WT-CLS1 showed any epithelial surface marker expression (Fig. 1B and C). CCG-99-11 showed a high CD56-NCAM expression whereas only a small minority of the WT-CLS1 cells, were positive for CD56-NCAM (Figs. 1B and C) and [5].

**Orthotopic Xenograft Models: Survival and Tumor Burden**

Orthotopic xenografts were established by implantation of WiT49 cells (n = 3), CCG-99-11 cells (n = 4), and WT-CLS1 cells (n = 4) into the kidney of immunodeficient NSG mice. The mice were monitored for signs of tumor burden. Orthotopic tumors were established in all mice. Mice injected with CCG-99-11 cells were sacrificed after 39 days (median) and WT-CLS1-injected animals were sacrificed after 90.5 days (median) (Fig. 1D). Mice with WiT49 injected cells showed no signs of tumor growth after more than three months, and after 141 days they were sacrificed; small intrarenal tumors of a few millimeters were then found (Fig. 1D). Analysis of xenograft tissue sections revealed that proliferation was very low in WiT49 tumors, with a mitotic index of zero, corroborated by a very low Ki67-positive cell fraction (Table I). In contrast, the CCG-99-11 xenografts were fast growing with 60–90 mitoses/10 HPF (high power fields; Table I), and WT-CLS1 xenografts showed an intermediate growth rate with a mitotic index of 30-50/10 HPF (Table I).

**Invasion Patterns and Tumor Spread**

Macro- and microscopic examination of orthotopic WiT49, CCG-99-11, and WT-CLS1 xenografts showed invasion of kidney parenchyma in all mice (Fig. 1E, Figs. 2A, B and G, and Table I). In WiT49-injected mice, we found no evidence of metastatic tumor cells in contralateral kidney, liver, or lungs. On the contrary, CCG-99-11 xenografts showed an aggressive phenotype with intrarenal blood vessel invasion (Fig. 2F) and metastases to the liver in all cases (Table I). Individual CCG-99-11-carrying mice presented with tumor invasion into either the adrenal gland, pancreas (Fig. 2D) or bone marrow, respectively (Table I). All WT-CLS1 mice exhibited intrarenal vascular invasion (Fig. 2I), and evidence of adrenal, pancreatic, and abdominal wall muscle invasion in some mice (Table I). Metastases to liver and lungs were found in all four WT-CLS1-tumor carrying mice (Table I and Figs. 1F and H). The spleens of WT-CLS1 mice were surrounded by tumor, but we found no evidence of actual tumor spread/invasion (Fig. 1G).

**Resemblance to WT Histology**

To group the xenografts according to their resemblance of classic triphasic WTs, xenograft sections were first analyzed for immature tubule formation (epithelial structures), blastemal and stromal components by routine histological staining (haematoxylin and eosin). In accordance with previous publications [1,3], WiT49, both as xenograft and in cell culture form (see above), displayed an epithelial phenotype with evident epithelial structures (Fig. 2C) corresponding to immature tubular formations, making out 7–30% of tumor volume (Table I). Tumor cysts were also visible in WiT49 xenografts but not in CCG-99-11 or WT-CLS1 xenografts. WiT49 xenografts also contained a small blastemal-like compartment and a large stromal compartment (Fig. 2C and Table I). Taken together, WiT49 tumors were epithelial with close to triphasic histology. The characteristics of CCG-99-11 xenografts diverged from WiT49, due to their predominantly blastemal-like small round blue cell morphology with rosette-like structures (Fig. 2E and Table I). WT-CLS1 xenografts showed features not very characteristic of WT morphology, growing in continuous sheets of pleomorphic cells with large pale cytoplasm (Fig. 2H), indicating an epithelial, rather than a blastemal phenotype, but with a complete absence of tubule formation and stroma, and only a minor cell population resembled classic-appearing blastema (Table I). All xenografts from all cell lines also showed multiple small foci with features of anaplasia, including variation in nuclear size and hyperchromatic nuclei (Supplementary Fig. 1). CCG-99-11 and WT-CLS1 also exhibited atypical mitoses, with a prevalence of multinuclear mitoses in tissue sections ranging from 1% to 6% in CCG-99-11 and 1% to 7% in WT-CLS1.

**Protein Expression of WT Markers**

To further evaluate the biologic behavior of the xenograft tumors, sections were stained by immunohistochemistry and immunofluorescence for clinically relevant WT markers such as WT1. A prominent WT1 nuclear staining pattern was evident in epithelial tubular structures of WiT49 xenografts (Fig. 3A and Table I), but not in other tissue elements. P53 was expressed in all histological compartments of WiT49 xenografts, confirming these cells as tumor cells and distinguishing them from mouse kidney host cells (inset Fig. 3A). Strong pan-cytokeratin AE1/3 (PanCK)
expression was found in all histological elements, including elements with a stromal morphology (Figs. 3B and D). CD56-NCAM and SIX1 were also detected in all elements of WiT49 xenografts (Figs. 3C and D). In CCG-99-11 xenografts, WT1 and PanCK staining was only weak and focal (Figs. 3E and F and Table I). The majority of the cells expressed CD56-NCAM; SIX1 expression, on the other hand, was weak and diffuse (Figs. 3G and H). Despite the absence of tubule formation, strong staining for PanCK (Fig. 3J) and regional positivity for WT1 (Fig. 3I), was seen in the orthotopic WT-CLS1 xenografts (Figs. 2G–I, Figs. 3I–L, and Table I). CD56-NCAM was only focally expressed along with diffuse weak positivity of SIX1 (Figs. 3K and L).
### TABLE I. Summary of Histology and Immunostainings of Wilms tumor xenografts

<table>
<thead>
<tr>
<th></th>
<th>WiT49 (n = 3)</th>
<th>CCG-99-11 (n = 4)</th>
<th>WT-CLS1 (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H–E stain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immature tubule formation</td>
<td>7–30%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Blastema</td>
<td>1–15%</td>
<td>90%</td>
<td>0–10%</td>
</tr>
<tr>
<td>Stromal component</td>
<td>40–85%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Invasion</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Metastases</td>
<td>Kidney (3)</td>
<td>Kidney (4), vessels (4), adrenal (1), pancreas (1), bone marrow (1)</td>
<td>Kidney (4), vessels (3), adrenal (1), pancreas (1), muscle (2)</td>
</tr>
<tr>
<td>Mitoses/10 HPF</td>
<td>–</td>
<td>Liver (4)</td>
<td>Lung (4), Liver (4)</td>
</tr>
<tr>
<td>IHC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CKA/E1/3</td>
<td>90%</td>
<td>0%</td>
<td>90%</td>
</tr>
<tr>
<td>CD56</td>
<td>70–80%</td>
<td>15–50%</td>
<td>2–15%</td>
</tr>
<tr>
<td>Ki67</td>
<td>1–2%</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>WT1</td>
<td>15–40%</td>
<td>0%</td>
<td>1–5%</td>
</tr>
<tr>
<td>p53</td>
<td>90%</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

H–E, hematoxylin and eosin; IHC, immunohistochemistry.

Fig. 2. Histological features in WT xenografts. A–C: Orthotopic WiT49 xenografts (i in A) in the mouse kidney (k in A), exhibited infiltrative growth surrounding murine kidney tubules (B). The tumors demonstrated triphasic features with immature epithelial tubule formation (e in C), stromal areas with dispersed tumor cells (s in C), and also foci of densely packed blastema-like cells (b in C). D–F: CCG-99-11 xenografts showed extensive extra-renal growth, exemplified by invasion of the pancreas (p in D), exhibited a predominantly blastema-like small round blue cell morphology (E) as well tumor emboli in vessels of the renal pelvis (F). G–I: WT-CLS1 xenografts (i in G) infiltrated the mouse kidney (k in G), grew predominantly as epithelioid sheets (H), also with vascular invasion in the renal pelvis (v in I).
DISCUSSION

Taken together we conclude that WiT49 orthotopic xenografts, which showed epithelial characteristics and close to triphasic histology, would represent the best in vivo model for the common mixed type WT in comparison to xenografts derived from CCG-99-11 and WT-CLS1 cells. Orthotopic WiT49 xenografts have previously been established in nude mice [1,2]. Here we used NSG mice, which are more immunocompromised than nude mice, thus expected to provide an even higher tumor take. Consistent with the present findings, WiT49 cells have not previously been shown to create metastatic disease, despite their origin from a lung metastasis [3]; even though NSG mice could potentially have been a permissive system for metastasis formation, due to their severe immunodeficiency. Li et al. [1] refer to WiT49 xenografts as biphasic with primarily a stromal and an epithelial component, while a blastemal component cannot be delineated. We observed a similar pattern in our xenografts. Although blastemal-like islands were observed in routine staining, which are reckoned to be the precursors of the more mature epithelial structures, these islands did not show a selective SIX1 positive/PanCK-negative immunoprofile as expected from regular WT blastema [5]. Also, our WiT49 xenografts revealed signs of anaplasia, in line with the xenografts produced by Bielen et al. [2]. Although confined to the kidney, which limits its usefulness in analyses of metastatic disease, it has a good resemblance to WT histology making it suitable for studies of WT histology and non-metastatic WTs. The resemblance to proper WTs in patients is further supported by the prominent WT1 staining, especially in epithelial structures, which has not been shown for WiT49 based orthotopic xenografts before. The WiT49 xenograft could be classified as a low stage WT with anaplastic mixed type histology (Table II) [6]. Worth noticing is the long incubation time for WiT49 xenografts, which we presented in this study (approximately 3 months), in comparison to previous publications on the subject [1,2]. The longer in vivo growth of WiT49 xenografts, compared to the other cell lines, probably better

![Image](image_url)
reflects WT formation in afflicted children, which supposedly takes years from congenital precursor lesions (nephrogenic remnants) to full blown tumor disease.

CCG-99-11 xenografts could serve as a model for blastemal WT [2]. However, it is puzzling that they did not show any strong staining for the blastemal marker SIX1, especially since the SIX1 protein is easily detected in vitro in these cells [5]. It can be speculated that the in vivo environment brings about a down-regulation of SIX1 in these specific cells. WT-CLS1 did not show distinct WT-like histological features. However, both WT-CLS1 and CCG-99-11 formed xenografts with metastatic disease (Table I) which placed these xenografts in the category of high stage tumors (Table II) [6]. In lack of other metastatic WT models, both CCG-99-11 and WT-CLS1 could be used for this specific purpose.

Continuously growing WT cell lines are known to be notoriously difficult to establish, not least from favorable histology WTs [7,8]. The few WT cell lines presented in the literature have mostly been established from anaplastic WTs, and are therefore, not representative of the majority of WTs [3,4,9,10]. In addition some WT cell lines which were first claimed to be of WT origin were later determined to be derived from other pediatric tumors [11,12]. This, taken together with the fact that the three cell lines studied in the present report had all grown for prolonged time in vitro, made them unlikely to be representative of the majority of WTs, which are triphasic favorable histology tumors. The limited retention of WT morphology in our orthotopic xenografts was therefore not unexpected.

In the hunt for personalized medicine a preferred WT model for patient specific drug evaluation would be based on orthotopic explants of primary tumors, in order to mimic the original tumor microenvironment [13]. However, few publications have addressed this issue in a WT context [13]. More common is the successful establishment of subcutaneous WT explant xenografts [14,15]. Wegert et al. [8] have been able to culture primary WT explants and it would be of great interest to assess whether those cultures were able to form orthotopic xenografts in mice. However, sample availability is very limited and the creation of a WT model based on WT cell lines might appear to be a useful alternative. In addition orthotopic xenografts based on cell lines are important for experiments where cell lines have been genetically modified in order to study the effect of a specific gene on tumor formation and progression.

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REFERENCES


TABLE II. Clinical Categorization of Wilms tumor xenografts

<table>
<thead>
<tr>
<th>Stage</th>
<th>Histology</th>
<th>“Outcome”</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT49</td>
<td>I or II</td>
<td>Mixed-anaplastic</td>
</tr>
<tr>
<td>CCG-99-11</td>
<td>IV</td>
<td>Blastemal-anaplastic</td>
</tr>
<tr>
<td>WT-CLS1</td>
<td>IV</td>
<td>Epithelial-anaplastic</td>
</tr>
</tbody>
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