Original Article

*N*-Methyl-*N*-nitrosourea-induced Renal Tumors in Rats: Immunohistochemical Comparison to Human Wilms Tumors

Katsuhiko Yoshizawa¹,*, Yuichi Kinoshita¹ ², Yuko Emoto¹ ², Ayako Kimura¹, Norihisa Uehara¹, Takashi Yuri¹, Nobuaki Shikata², and Airo Tsubura¹

¹ Department of Pathology II, Kansai Medical University, Hirakata, Osaka 573-1010, Japan
² Division of Pathology, Kansai Medical University Takii Hospital, Moriguchi, Osaka 570-8507, Japan

Abstract: *N*-Methyl-*N*-nitrosourea (MNU)-induced renal tumors in rats and Wilms tumors in humans were compared. Renal mesenchymal tumors (RMTs) and nephroblastomas (blastemal and epithelial components) in female Lewis rats treated with a single intraperitoneal injection of 50 mg/kg MNU at birth and Wilms tumors (blastemal, epithelial and mesenchymal components) in humans were analyzed for the expression of pancytokeratin (CK), vimentin, p63, α-smooth muscle actin (SMA), desmin, S100, CD117/c-kit, Wilms tumor 1 protein (WT1) and β-catenin. The mesenchymal components of rat RMTs and human Wilms tumors expressed vimentin, SMA and β-catenin. The blastemal components of rat nephroblastomas and human Wilms tumors expressed vimentin, CD117/c-kit and β-catenin. The epithelial components of rat nephroblastomas and human Wilms tumors expressed vimentin and β-catenin. WT1 was expressed in different cellular components of rat tumors as compared with human Wilms tumors; the expression was seen in mesenchymal tumors and blastemal components of nephroblastomas in rats and epithelial components in human Wilms tumors. CK, p63 and CD57 were not expressed in rat RMTs or nephroblastomas, while CK and WT1 were expressed in epithelial components and CD57 was expressed in blastemal and epithelial components of human Wilms tumors. Rat and human tumors were universally negative for the expression of desmin and S100. The immunohistochemical characteristics of rat renal tumors and human Wilms tumors may provide valuable information on the differences in renal oncogenesis and biology between the two species. (DOI: 10.1293/tox.26.141; J Toxicol Pathol 2013; 26: 141–148)

Keywords: immunohistochemistry, kidney, *N*-methyl-*N*-nitrosourea, nephroblastoma, renal mesenchymal tumor, Wilms tumor

Introduction

Wilms tumor (nephroblastoma) accounts for nearly 6% of all pediatric cancers and more than 95% of all kidney tumors in children, and it afflicts 1 in 10,000 children.² Principle risk factors for renal cancer include inherited germine mutations, and a number of loci involved in the development of Wilms tumor have been characterized in humans. The key locus is WT1, a tumor suppressor gene located on chromosome 11p. Wild-type WT1 is overexpressed in approximately 90% of Wilms tumors.³

*N*-Methyl-*N*-nitrosourea (MNU), a direct-acting alkylating agent that interacts with DNA, is toxic and carcinogenic to the immune, hematopoietic, reproductive, mammary, dentine, gastrointestinal, pancreatic, nervous and/or sensory systems. Nitrosourea compounds including *N*-methyl-*N*-nitrosourea (MNU) also possess carcinogenic potency in the kidneys,⁵ and MNU induces renal mesenchymal tumors (RMTs) and nephroblastomas in rats.⁶ The formation and persistence of DNA adducts, such as O⁶-methylguanine, and K-ras codon 12 and 16 point mutations in renal cortical tubular cells and/or mesenchymal interstitial cells is related to the tumor development induced by alkylating agents.⁷

Few reports have compared in vivo data between rat and human renal tumors.¹⁴,¹⁵ The present study compares the immunohistochemical characteristics of rat mesenchymal tumors and nephroblastomas induced by MNU and human Wilms tumors.

Materials and Methods

Animal procedures

The study protocol and all animal procedures were approved by the Animal Care and Use Committee of Kansai Medical University and were in accordance with the guidelines for animal experimentation at Kansai Medical University. One-week pregnant SPF/VAF rats [LEW/Crl(Crlj)] were purchased from Charles River Laboratories Japan (Yokohama, Japan). At birth, pups (n=33) received a single
intrapерitoneal (i.p.) injection of 50 mg/kg MNU. Fourteen surviving female rats were used for the analysis at the age of 16 weeks. Rats were maintained under specific pathogen-free conditions and had free access to water and a CE-2 diet (CLEA Japan, Tokyo, Japan) according to a previous study12. Animals were housed in plastic cages with paper-chip bedding (Paper Clean, SLC, Hamamatsu, Japan) and sacrificed by exsanguination via aortic transection. All pups were observed daily for clinical signs of toxicity and were weighed at the time of MNU treatment and formation, or mature epithelial ducts16, 20.

Histopathological examination

Renal tissues were fixed overnight in 10% neutral buffered formalin, embedded in paraffin, sectioned at a thickness of 4 μm and stained with hematoxylin and eosin (HE). Histopathological terminology and diagnostic criteria for rodent renal neoplastic lesions were in accordance with the International Harmonization Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice Project16. Human terminology and diagnostic criteria for renal neoplastic lesions were in accordance with the guidelines of the Armed Forces Institutes of Pathology17. Rat RMTs originated from foci of atypical fibroblast-like cells in the interstitium of the outer stripe of the outer medulla, similar to congenital mesoblastic nephromas in human infants18, 19. The rat RMTs are characterized by a heterogeneous connective tissue cell composition with a predominance of spindle cells with a primitive mesenchyme and smooth muscle fibers and occasional rhabdomyoblasts, striated muscle, cartilage and osteoid or hemangiosarcomatous areas16, 20.

Nephroblastoma in rats, equivalent to Wilms tumor in human nomenclature, originates from the metanephric blastema. It is characterized by discrete clusters of highly basophilic blast cells surrounding mature ducts and organoid differentiation as epithelial rosettes, primitive basophilic tubules, attempted glomerulus formation, or mature epithelial ducts16, 20.

Immunohistochemical analysis

Sequential sections of nodules were labeled with antibodies to pancytokeratin (CK) as an epithelial cell marker21, vimentin as a mesenchymal cell marker, p63 as a basal/muscular cell marker22, α-smooth muscle actin (SMA) and desmin as muscular cell markers, S-100 as a Schwann cell and adipocyte marker21, CD57 as a renal pelvic epithelial cell/Wilms tumor cell marker23, CD117/c-kit as a gastrointestinal stromal tumor/Wilms tumor cell marker24 and Wilms tumor 1 protein (WT1) and β-catenin as Wilms tumor cell marker25. Detailed staining protocols for all antibodies are listed in Table 1. Briefly, sections were deparaffinized, hydrated and blocked for endogenous peroxidase. Heat-induced epitope retrieval was performed for all antibodies except SMA, desmin and S-100. The antigen-antibody complexes were identified by using a streptavidin-biotin (LSAB) staining kit (Dako) according to the manufacturer’s instructions. Antigen retrieval was conducted by pressure-cooker heating (Pascal, Dako).

### Table 1. Immunohistochemistry Methods

<table>
<thead>
<tr>
<th>Primary antibody tested</th>
<th>Source</th>
<th>Clone</th>
<th>Working dilutions</th>
<th>Antigen retrieval</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK</td>
<td>Dako</td>
<td>AE1/AE3</td>
<td>1:50</td>
<td>115°C for 10 min</td>
<td>Epithelial cell</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Dako</td>
<td>V9</td>
<td>1:50</td>
<td>115°C for 10 min</td>
<td>Mesenchymal cell</td>
</tr>
<tr>
<td>p63</td>
<td>Thermo</td>
<td>4A4</td>
<td>1:100</td>
<td>115°C for 10 min</td>
<td>Basal cell / muscular cell</td>
</tr>
<tr>
<td>SMA</td>
<td>Dako</td>
<td>1A4</td>
<td>1:500</td>
<td>-</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>Desmin</td>
<td>Dako</td>
<td>D33</td>
<td>1:50</td>
<td>-</td>
<td>Striated muscle cell</td>
</tr>
<tr>
<td>S-100</td>
<td>Dako</td>
<td>Polyclonal</td>
<td>1:500</td>
<td>-</td>
<td>Schwann cell/adipocyte</td>
</tr>
<tr>
<td>CD57</td>
<td>Novo</td>
<td>NK-1</td>
<td>1:50</td>
<td>0.1% trypsin for 60 min at 37°C</td>
<td>Renal pelvic epithelial cell/</td>
</tr>
<tr>
<td>CD117/c-kit</td>
<td>Nichirei</td>
<td>Polyclonal</td>
<td>Prediluted</td>
<td>115°C for 10 min</td>
<td>Gastrointestinal stromal tumor/Wilms tumor cell</td>
</tr>
<tr>
<td>WT1</td>
<td>Nichirei</td>
<td>6F-H2</td>
<td>Prediluted</td>
<td>115°C for 10 min</td>
<td>Wilms tumor cell</td>
</tr>
<tr>
<td>β-catenin</td>
<td>BD</td>
<td>14/β-Catenin</td>
<td>1:100</td>
<td>115°C for 10 min</td>
<td>Wilms tumor cell</td>
</tr>
</tbody>
</table>

1) CK, pancytokeratin; SMA, α-smooth muscle actin; WT1, Wilms tumor 1 protein; p63 Dako (Carpinteria, CA, USA); Thermofisher Scientific (Fremont, CA, USA); Novocastra (Newcastle upon Tyne, UK); Nichirei Bioscience (Tokyo, Japan); BD Transduction Laboratories (Franklin Lakes, NJ, USA). 2) Each primary antibody was reacted for 1 hr at room temperature. The antigen-antibody complexes were identified by using a streptavidin-biotin (LSAB) staining kit (Dako) according to the manufacturer’s instructions. 3) Antigen retrieval was conducted by pressure-cooker heating (Pascal, Diko). - Antigen retrieval was not conducted.
p63, vimentin, SMA, desmin and S-100. Three representative rat tumors and human Wilms tumor specimens were examined immunohistochemically for CD57, CD117/c-kit, WT1 and β-catenin. The intensity of immune staining was scored as negative (−), weak (+), moderate (+++) or strong (+++). Histopathological examination including immunohistochemical scoring was conducted by two toxicologic pathologists certified by the Japanese Society of Toxicologic Pathology (K.Y., A.T.) and by two human pathologists certified by the Japanese Society of Pathology (N.S., A.T).

Results

At 16 weeks of age, 10 RMTs and 3 nephroblastomas developed in the 14 surviving female rats that were treated with 50 mg/kg MNU at birth. All surviving female rats developed mammary cancers. RMTs were predominantly composed of atypical spindle cells with areas of heterogeneous connective tissue surrounding non-tumorous renal tubules (Fig. 1a). Nephroblastomas showed a biphasic pattern with foci of highly basophilic blast cells (blastemal components) and organoid differentiation such as epithelial rosettes, primitive basophilic tubules and glomerulus formation (epithelial components) (Fig. 1b). In contrast, human Wilms tumors showed a triphasic morphology composed of mesenchymal (Fig. 1c), blastemal (Fig. 1d), and epithelial (Fig. 1e) components in all specimens examined. The formation of striated muscle, cartilage and osteoid or hemangiosarcomatous areas was not seen in any of the rat or human tumors.

The results of immunohistochemical analysis for rat RMTs and nephroblastomas and human Wilms tumors are summarized in Table 2. Each RMT, nephroblastoma and Wilms tumor revealed similar results of immunohistochemical analysis, respectively. In rat RMTs and nephroblastomas, no signals for CK were seen in the cytoplasm of any type of tumor cells, in contrast to the positive signals in residual normal renal tubules within the tumors. Strong positive signals for vimentin were seen diffusely in the cytoplasm of mesenchymal tumor cells (Fig. 2a). In nephro-
Comparison of Rat Renal Tumor and Human Wilms Tumor

**Table 2. Immunohistochemical Expression of Each Antigen in Rat and Human Renal Tumors**

<table>
<thead>
<tr>
<th>Primary antibody 1)</th>
<th>Rat Renal mesenchymal tumor 2)</th>
<th>Nephroblastoma 2)</th>
<th>Human Wilms tumor (Nephroblastoma) 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blastema</td>
<td>Epithelium</td>
<td>Mesenchymal</td>
</tr>
<tr>
<td>CK</td>
<td>–</td>
<td>–</td>
<td>–/4)</td>
</tr>
<tr>
<td>Vimentin</td>
<td>++/5)</td>
<td>++</td>
<td>+/5)</td>
</tr>
<tr>
<td>p63</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>SMA</td>
<td>+/5)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Desmin</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>S-100</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CD57</td>
<td>–</td>
<td>–</td>
<td>–/4)</td>
</tr>
<tr>
<td>CD117/c-kit</td>
<td>–</td>
<td>–/4)</td>
<td>–/4)</td>
</tr>
<tr>
<td>WT1</td>
<td>–</td>
<td>+/5)</td>
<td>–/4)</td>
</tr>
<tr>
<td>β-catenin</td>
<td>–</td>
<td>–/4)</td>
<td>–/4)</td>
</tr>
</tbody>
</table>

1) CK, pancytokeratin; SMA, α-smooth muscle actin; WT1, Wilms tumor 1 protein. 2) Ten renal mesenchymal tumors and 3 nephroblastomas in rats and 3 human Wilms tumor specimens were examined immunohistochemically for CK, p63, vimentin, SMA, desmin, and S-100. Human Wilm tumors specimens were obtained from two patients. Three representative rat tumors and human Wilms tumor specimens were examined immunohistochemically for CD57, CD117/c-kit, WT1, and β-catenin. 3) The intensity grade of immune staining was scored as follows: –, negative; +, weak; ++, moderate; or ++++, strong. 4) Positive cells were focally seen or scattered in the component. 5) Positive cells were diffusely seen in the component.

Blastemas, blastemal cells were also moderately positive, and the positivity was seen diffusely. Some epithelial foci and ductal structures were weakly positive for vimentin (Fig. 2b). Some areas of RMTs were moderately positive for SMA in the cytoplasm of tumor cells, and the positivity was seen diffusely (Fig. 2c). No blastemal or epithelial cells in any areas of the nephroblastomas were positive for SMA.

The neoplastic cells of mesenchymal tumors were negative for CD117/c-kit. Some foci of CD117/c-kit-positive cells were seen in the blastemal components of nephroblastomas. The signals were located in the cytoplasm (Fig. 2d). However, many blastemal cells and epithelial components were negative for CD117/c-kit. Mesenchymal tumor cells showed weak positivity for WT1, which was detected focally and mainly in the nuclei (Fig. 2e). Blastemal cells of nephroblastomas possessed similar characteristics (Fig. 2f). No signals for WT1 were seen in the epithelial cells of any components. The β-catenin signals were diffuse but weak in the cytoplasm of mesenchymal tumor cells (Fig. 2g). Slight to moderate expression of β-catenin was seen in the cytoplasm of tumor cells in the blastemal and epithelial components of nephroblastomas (Fig. 2h). The positive cells were located diffusely in the blastemal component. In contrast, the tumor cells of the epithelial component were focally positive, as well as the positivity in the normal renal tubular epithelia. No tumor cells were positive for p63, S100, desmin or CD57 in any components of the rat RMTs or nephroblastomas.

In human Wilms tumors, CK was strongly expressed in the cytoplasm of epithelial components, and the positivity was seen diffusely. In contrast, tumor cells of the blastemal and mesenchymal components were basically negative for CK. Some CK-positive cells were present in the blastemal component, suggesting transformation into epithelial cells (Fig. 3a). Strong expression of vimentin was diffusely seen in the cytoplasm of all mesenchymal cells (Fig. 3b). Blastemal cells were moderately positive, and some epithelial foci and ductal structures were slightly positive for vimentin. The nuclei of some mesenchymal cells were positive for p63 (Fig. 3c); however, no signals were seen in tumor cells of the blastemal and epithelial components. Some areas of mesenchymal components expressed SMA in the cytoplasm as well as the walls of supporting vascular structures (Fig. 3d). No blastemal or epithelial cells were positive for SMA in any areas of the tumors.

CD57-positive epithelial cells and blastemal cells were present in the epithelial and blastemal components, respectively (Fig. 3e). No mesenchymal cells were positive for CD57 in any components. Although many blastemal cells were negative for CD117/c-kit, some foci of positive cells in the cytoplasm were seen in blastemal components (Fig. 3f). No signals were seen in epithelial or mesenchymal components. Weak expression of WT1 was detected focally in the epithelial components, such as the epithelial foci and ductal structures (Fig. 3g). No WT1 signals were present in the cytoplasm of blastemal and mesenchymal cells. Moderate to strong expression of β-catenin was present in the cytoplasm of tumor cells in the blastemal and epithelial components, respectively (Fig. 3h). The positive cells were located diffusely in both components. The focal area of mesenchymal components contained weakly positive cells. No tumor cells were positive for S100 or desmin in any components of the human Wilms tumors.

**Discussion**

In the present study, rat RMTs and nephroblastomas induced by MNU and human Wilms tumors were compared morphologically and immunohistochemically. Wilms
tumors are embryonic renal neoplasms believed to result from a perturbation in the development of the metanephric blastema, and they have a bi- or triphasic morphology comprised of blastemal, mesenchymal and epithelial cells. Morphologically, rat nephroblastomas showed a biphasic morphology composed of blastemal and epithelial components, while human Wilms tumors showed anaphasic morphology composed of blastemal, epithelial and mesenchymal components. An immunohistochemical analysis for several antigens (CK, vimentin, p63, SMA, desmin, S-100, CD57, CD117/c-kit, WT1 and β-catenin) expressed in human Wilms tumors was performed. The proliferating spindle tumor cells in rat RMTs were positive for vimentin, SMA, WT1 and β-catenin. Additionally, the mesenchymal components in human Wilms tumors revealed similar tendencies, except that they were positive for p63 and negative for WT1. The blastemal cells of rat nephroblastomas were positive for vimentin, CD117/c-kit, WT1 and β-catenin, while CK, vimentin, CD57, CD117/c-kit and β-catenin were expressed in the blastemal components of Wilms tumors. The epithe-

Fig. 2. Immunohistochemistry for rat renal mesenchymal tumor (RMT) and nephroblastoma. Vimentin expression in RMT (a) and nephroblastoma (b), α-smooth muscle actin expression in RMT (c), CD117/c-kit expression in nephroblastoma (d), WT1 expression in RMT (e) and nephroblastoma (f), and β-catenin expression in RMT (g) and nephroblastoma (h). Bar = 100 μm.
Comparison of Rat Renal Tumor and Human Wilms Tumor

Immunohistochemistry for human Wilms tumor. Pancytokeratin expression in blastemal component (a), vimentin expression in mesenchymal component (b), p63 expression in mesenchymal component (c), α-smooth muscle actin expression in mesenchymal component (d), CD57 expression in blastemal component (e), CD117/c-kit expression in blastemal component (f), WT1 expression in epithelial component (g), and β-catenin expression in blastemal component (h). Bar = 100 μm.

In a previous study of dimethylnitrosamine-induced mesenchymal tumors in F344 rat kidneys, proliferating neoplastic cells had strong vimentin positivity with desmin expression but without S100 expression. In rat renal tumors including RMTs, the concentration and expression of S100 protein changes compared with normal renal tissue. In the present study, no cellular components of rat RMTs or nephroblastomas possessed any signals for desmin, Schwann cells or adipocytes. In contrast, mesenchymal components of rat nephroblastomas and human Wilms tumors were positive for vimentin and β-catenin, and CK, CD57 and WT1 were expressed in the epithelial components of Wilms tumors.
components of human Wilms tumors were weakly positive for p63. p63 immunohistochemistry is a useful marker for muscle differentiation through the intermediation of the retinoblastoma protein, and rhabdomyomatous components of Wilms tumors are positive for p63 even when desmin protein is not expressed. Therefore, some areas of mesenchymal components in our human cases might differentiate into rhabdomyocytes.

In human Wilms tumors, CD57 is expressed in blastemal (55% cases), mesenchymal (18% cases), and epithelial components (76% cases). Therefore CD57 immunohistochemistry is a good marker for the diagnosis of Wilms tumors. In the present research, no signals were detected in rat mesenchymal tumors and nephroblastomas, in contrast to human Wilms tumors. Signals for CD117/c-kit were detected in the blastemal components of rat nephroblastomas and human Wilms tumors. CD117/c-kit plays an important role in the development and survival of many cell types, and mutations of the CD117/c-kit gene cause constitutive ligand-independent activation of the KIT receptor and upregulation of cellular growth. In 16 of 40 Wilms tumors, CD117/c-kit expression was detected focally and with patch distribution (>10% of neoplastic elements) as a strong membranous/cytoplasmic reactivity in epithelial and blastemal elements. From the results of our research, CD117/c-kit may be a good marker for blastemal cells of rat renal tumors.

WT1 is coordinately expressed and strongly associated with the differentiation of metanephric blastemal cells into epithelial cells. The upregulation of WT1 is required during the mesenchymal-epithelial transition, showing renal epithelial differentiation in mesenchymal cells. WT1 is expressed only in neoplastic structures whose normal counterparts also express WT1 and, and immunohistochemical detection of WT1 is traditionally used as a diagnostic marker for Wilms tumor. In the present study, WT1 signals in rat tumors were seen in different cellular components from human Wilms tumors; RMTs and blastemal components of rat nephroblastomas expressed WT1 mainly in the nuclei, and epithelial components of human Wilms tumors expressed WT1 in the nuclei and cytoplasm. In general, the signals are located basically in the nuclei of human Wilms tumor cells. However, a recent report provided evidence that WT1 is involved not only in transcriptional regulation in the nuclei but also in RNA metabolism and translational regulation in the cytoplasm; as a result, cytoplasmic expression of WT1 has been shown in a large proportion of human cancers.

The genetic aberrations underlying Wilms tumor development can include inactivating mutations of the WT1 gene or the WT gene on the X chromosome (WTX) and stabilizing/activating mutations of β-catenin (CTNNB1). There is strong evidence that the Wnt/β-catenin pathway is important in the development of human Wilms tumors. Immunohistochemical analysis of β-catenin expression in chemically induced rat nephroblastomas including the mesenchymal cell type revealed that 64% of tumors are positive for nuclear accumulation of β-catenin protein, showing the same phenomenon as 66% of human Wilms tumors in Kras<sup>Δex3</sup>/Catnb<sup>−/−</sup> mice treated with 1.5 mg tamoxifen from postnatal days 1 to 5, as a human Wilms tumor mouse model, exhibit strong β-catenin expression and nuclear translocation. In our study, β-catenin signals were expressed in the cytoplasm of all components of rat and human renal tumors, although the nuclei exhibited negative staining.

In summary, the mesenchymal components of rat RMTs and human Wilms tumors expressed vimentin, SMA, and β-catenin. The blastemal components of rat nephroblastomas and human Wilms tumors expressed vimentin, c-kit, and β-catenin. The epithelial components of rat nephroblastomas and human Wilms tumors were positive for vimentin and β-catenin. WT1 signals in rat tumors were seen in different cellular components from human Wilms tumors, probably due to the stage of the mesenchymal-epithelial transition. No signals for CK, p63 or CD57 were seen in rat mesenchymal tumors and nephroblastomas, unlike human Wilms tumors. The different results of immunohistochemical analysis between rat and human renal tumors may depend on the growth of the tumors examined, as well as the differentiation of tumor cells. Additional immunohistochemical analyses are needed to understand the biology of chemically induced renal tumors in rats; however, the present research might provide valuable information on rat renal tumors by comparison of immunohistochemical characteristics with those of human Wilms tumors.

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