Human Sarcomatous Wilms’ Tumor Lines: Evidence for Epithelial Differentiation in Clear Cell Sarcoma of the Kidney

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ABSTRACT

The biological nature of human sarcomatous Wilms’ tumor (SWT) was studied by analyzing newly established SWT lines, both heterotransplantable in nude mice and cultured in vitro. Five lines in nude mice include two from clear cell sarcoma of the kidney (CCSK), two from malignant rhabdoid tumor of the kidney (MRTK), and one from unclassified sarcoma. Five in vitro lines include three from CCSK, one from MRTK, and one from unclassified sarcoma. All of these in vitro cell lines produced tumors when inoculated in nude mice. Most of lines, especially of MRTK and unclassified sarcoma, well maintained their original morphological characteristics. However, CCSK lines, both heterotransplantable and in vitro, often showed unique morphological changes such as the increase of cells with eosinophilic cytoplasm and the production of mucin. Ultrastructurally, clusters of intermediate filaments, twisted sheaves of filaments resembling tonofilaments, intermediate junctions, and intracellular canaliculi were found in these cells. These findings suggested that CCSK had the latent epithelial nature which became obvious in the cell lines. This was confirmed by immunohistochemical and immunoblotting analyses with anticytokeratin antibodies. The result proved that CCSK expressed cytokeratin 8 (M, 52,000) and 19 (M, 40,000) as well as nephroblastic Wilms’ tumor and strongly indicated that there was a close relationship between CCSK and nephroblastic Wilms’ tumor.

INTRODUCTION

There has been remarkable improvement in the management of NBW by group studies such as NWTS (1–4). Whereas, unfavorable renal tumors of unknown origin with histologically monomorphic growing appearance are actually still present in childhood (1–6). We tentatively termed them SWT.

Typical subtypes of SWT are CCSK (7, 8) and MRTK (9, 10). CCSK is also known as bone metastasizing renal tumor of the kidney because of a prediction for bone metastasis (11). MRTK is a term used to describe tumors resembling rhabdomyosarcoma but showing no evidence of skeletal muscle differentiation (1). These tumors have been classified in unfavorable variants of Wilms’ tumors (1, 2). However, MRTK is recently dropped out from NWTS-4 (4), because of identical tumors being found in various extrarenal sites (12, 13). Other sarcomatous tumors have been also reported such as US proposed by Gonzalez-Crussi (14). US is considered to be a very primitive undifferentiated malignancy and has none of the characteristic findings seen in CCSK or MRTK.

The individual nature of these subtypes and the relationship

Amoung them have been unsatisfactorily revealed. Furthermore, although the histogenesis of NBW has been well documented including our own (15), that of SWT is still controversial despite many hypotheses for their origins being built up (7–10). In order to clarify the nature of SWTs, we have established SWT lines heterotransplated in nude mice as well as in culture in vitro. In this study, by morphological, immunohistochemical, and immunoblotting analyses, we describe the unique biological property of CCSK not found in other SWTs.

MATERIALS AND METHODS

Tissues. Eight cases of human SWT were examined and are summarized in Table 1. Tumor tissues were obtained at surgical operations. Light microscopically, all of these showed diffuse monophasic and randomly arranged growth pattern without any organoid elements found in NBW except entrapped tubules. In Cases 1 to 5, most tumor cells had clear or vacuolated cytoplasm and vesicular nuclei. Distinctive arborizing vascular channels (2) were also found. These were diagnosed as being CCSK. Cases 6 and 7 had tumor cells with characteristic eosinophilic inclusions in the cytoplasm and prominent nucleoli in relatively large nuclei (1). These were diagnosed as being MRTK. Tumor cells of Case 8 had more abundant cytoplasm than CCSK cells but no intracytoplasmic eosinophilic inclusions (14). This was diagnosed as being US.

Seventeen cases of NBW, including 13 surgically resected tumors and four transplantable tumor lines in nude mice, were also examined. Characterization of two transplantable NBWs (OW, OW,) was already described (16). Kidney nephrons were obtained from two legal abortuses each of 12 and 23 weeks gestation estimated by crown-to-rump length. Establishment of Transplanted Tumor Lines in Nude Mice and of Cultured Cell Lines in vitro. Six of eight surgically resected tumors (Cases 1–3, 6–8) could be prepared for heterotransplantation according to the method described by J. Hata et al. (17). Thus, tissue fragments were s.c. transplanted into the backs of BALB/c nude mice. Mice were housed under germ free conditions and the successive transfer was performed by the same procedure.

Specimens for in vitro cultures were minced in the medium constituted of:1) mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 (GIBCO, Grand Island, NY) containing 10% fetal calf serum (Piton, Victoria, Australia) and inoculated into dishes. After growing enough, these were detached by pipetting and reseeded in appropriate dilutions. Subculturing was performed twice a week by the same method.

Chromosome numbers of in vitro cell lines were analyzed at the 10th passage. Cells cultured in colcemid-added medium were exposed to 0.075 M KCl and fixed in methanol/acetic acid solution. Metaphase spreads were prepared by dropping a cell suspension onto slides and were stained with Giemsa.

Monoclonal Antibodies. Mouse monoclonal antibodies used in this study were as follows. Two anticytokeratin antibodies were used. One was K8.13 (Bio Makor, Rehovot, Israel) recognizing cytokeratin 1, 5, 6, 7, 8, 10, 11, 18 (18) and another was MAK (TBI, Alamada, CA) reactive with cytokeratin 8, 14, 15, 16, 18, 19 (19). Antivimentin antibody was M725 (Dakopatts, Glostrup, Denmark). Anti-B-cell-associated antigen (CD 24) antibody, BA-1 (Hybritech, San Diego, CA), was also used because of its reactivity with metanephroblastic cells both in normal fetal kidneys and in NBWs (15, 20).

Immunohistochemistry. Nonfixed frozen sections were prepared for indirect immunoperoxidase staining (15). Sections were fixed in acetone and incubated sequentially with the primary and the secondary antibod-
ies. The latter was rabbit anti-mouse immunoglobulin antibody conjugated to horseradish peroxidase (Dakopatts, Grostrup, Denmark). Color reaction was carried out in Karnovsky solution containing 3,3'-diaminobenzidine and hydrogen peroxide (H$_2$O$_2$).

**Gel Electrophoresis and Immunoblotting of Cytokeratin Proteins.** For one-dimensional sodium dodecyl sulfate-polyacrylamide gel (8%) electrophoresis, lysates of cultured cells and tumor tissues were prepared with 1% Triton X100 and 1 mM protease inhibitor (21). After transfer of the proteins onto nitrocellulose sheets, free binding sites were blocked with bovine serum albumin. After incubation with K8.13 and MAK 6, the nitrocellulose blots were stained with the secondary antibody described above. Color reaction was done with o-dianisidine and H$_2$O$_2$.

### RESULTS

**Establishment of Heterotransplantable SWT Lines in Nude Mice.** Five out of six transplanted cases could successfully develop serially transplanted lines in nude mice (Table 1). All these lines grew in a diameter of approximately 1.5 cm by 4-8 weeks after every inoculation, and generally maintained their original histological features in repeated passages.

CR-SW3, derived from CCSK case 1, showed diffuse monophasic growing of polygonal cells with vesicular nuclei and poorly stained cytoplasms (Fig. 1A). Ultrastructurally, these cells showed electron lucent cytoplasms containing poorly developed cytoplasmic organelles which were similar to those of original tumor cells (Fig. 1B).

On the other hand, CR-SW5, another CCSK line derived from Case 2, showed mainly similar but occasionally different histological features from the original tumor. Thus, a myoid appearance was occasionally identified where tumor cells had more abundant cytoplasms (Fig. 1C and D). Ultrastructurally, these cells had increased number of cytokeratin organelles and sometimes contained the cluster of intermediate filaments (approximately 4–6 nm in diameter). Furthermore, some cells revealed the epithelial features such as twisted sheaves of filaments like tonofilaments, intermediate junctions (zonula adherens), and the intracellular canalici (Fig. 1E). All these findings were not shown in the original tumor tissue.

Both CR-SW1 and SW6 established from MRTK Cases 6 and 7, respectively, showed diffuse monophasic growth with intracytoplasmic eosinophilic inclusions which were frequently seen in original MRTK tumors (Fig. 2A). Ultrastructurally these distinctive inclusions consisted of a whorled mass of intermediate filaments (4–6 nm) (Fig. 2B).

CR-SW2, derived from US (Case 8), also showed monotonous proliferation (Fig. 2C). Ultrastructurally these cells had scant cytoplasmic organelles giving an impression that they were more primitive than other lines (Fig. 2D), consistent with the feature of original cells.

**In Vitro Cell Lines.** Five in vitro SWT cell lines were established (Table 2). All of these had been stably growing in tissue culture for more than 308, 263, 173, 92, and 57 passages for NCR-W1, W2, W3, W4, and W5, respectively.

NCR-W1, W3, and W5 were derived from CCSS, Cases 1, 2, and 5, respectively. NCR-W1 and W3 grew attached on the bottom of plastic flasks with polygonal shape for W1 and with spindle shape for W3. NCR-W5 grew as floating aggregates in the medium. Ultrastructurally these three lines exhibit similar appearance. Thus, cells had well-developed microvilli and abundant cytoplasmic organelles (Fig. 3A). Twisted sheaves of filaments resembling tonofilaments were occasionally found, especially in NCR-W1 cells (Fig. 3B). Of note, these features were not found in their original tumors.

NCR-W4 cells, from MRTK Case 7, grew both in attached form and in floating form. The characteristic whorled mass of intermediate filaments could be frequently identified by electron microscopy (Fig. 3, C and D), consistent with the finding in the original tumor cells.

NCR-W2 was derived from a transplanted US tumor line in nude mice (CR-SW2) and proved human origin by chromosome analysis. These cells loosely attached in round shapes. These had electron lucent cytoplasms and poorly developed organelles, consistent with the features found in original tumor.

Modal chromosome numbers were analyzed at the 10th passage. Those of NCR-W1, W2, W3, W4, and W5 were 45, 45, 46, 91, and 46, respectively. These chromosomes were all human types and have not changed in number despite repeated passages.

All of these SWT lines in vitro were found to produce tumors when 1 x 10$^7$ cells were s.c. injected into nude mice. Tumorigenicity was confirmed by repeated experiments. Histological features were almost same as transplanted lines from these original tumors.

**Immunohistochemistry.** Distribution of intermediate filaments dramatically changed in developing nephrons of human fetal kidney (Table 3). Metanephric blastemal were positive for vimentin, whereas tubular structures which eventually develop into renal tubules expressed cytokeratin but not vimentin. Interestingly, this distribution also changed with formation of glomeruli as shown in Fig. 4, A and B. In this stage, cytokeratin gradually lost its expression in lower limbs of S-bodies and vimentin started to appear again in the same part. Mature glomeruli were negative for cytokeratin but positive for vimentin. Thus, there are two stages when cytokeratin and vimentin may be coexpressed; the tubular formation from the metanephric blastema and the glomerular formation from the lower limbs of S-bodies.

In CCSK, the different positivity of cytokeratin and vimentin was obtained when primary tumors, heterotransplantable tumors, and in vitro lines were compared. Thus, all primary CCSK tumors expressed vimentin, but cytokeratin was not detected.
However, CCSK lines, heterotransplantable and in vitro, showed positivity both for cytokeratin and vimentin (Fig. 4, C and D). Such a diversity of the expression of intermediate filaments were not found in MRTKs nor in US. MRTK tumors, primary and lines (heterotransplantable and in vitro), were positive both for cytokeratin and vimentin. US tumors only expressed vimentin.

For comparison, expression of intermediate filaments in NBWs were also studied. Cytokeratin was positive only on epithelial elements, whereas vimentin was positive on blastemal
and stromal elements (Table 3). This distinct distribution in NBW was quite comparable to that of fetal kidney.

When the expression of CD24, recognized by BA-1, was studied, it was found that CD24 was totally negative in all samples from CCSK, MRTK, and US. However, it was positive on blastema! and epithelial components with the exception of glomerular epithelia in human fetal kidney (Table 3) (15).

Western Blotting of Cytokeratin. The results of immunohistochemical analyses suggest that the appearance of cytokeratin in CCSK tumor lines might be related to the morphological alternation to epithelioid nature. In order to clarify this point, cytokeratin species in CCSK and MRTK tumor lines were studied and were compared with those in NBW. As shown in Fig. 5, CCSK tumor lines, both heterotransplantable (CR-SW5) and in vitro (NCR-W1 and W3), had M, 52,000 and 40,000 cytokeratins which correspond to cytokeratins 8 and 19, respectively. Weak but similar expression was also found in other CCSK lines. Quite interestingly, identical cytokeratin species

<table>
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<tr>
<th>Cases</th>
<th>Subtypes</th>
<th>Name of lines</th>
<th>Passages</th>
<th>Doubling time (h)</th>
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<tr>
<td>1</td>
<td>CCSK</td>
<td>NCR-W1</td>
<td>308</td>
<td>60.4</td>
<td>43-48 (45)</td>
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<td>92</td>
<td>26.5</td>
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<td>263</td>
<td>27.3</td>
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*a* Mean. Three flasks were seeded for determination of doubling time.

b* 1 X 10⁶ cells were s.c. injected into the back of nude mice. Number of mice examined were six to eight.

*NE, not established.*
Fig. 3. Electron micrographs of CCSK and MRTK cell lines in vitro. A, an NCR-W1 (CCSK) cell has well-developed microvilli and intermediate filaments (x10,250); B, twisted sheaves of filaments resembling tonofilaments of an NCR-W1 cell (x14,000); C, an NCR-W4 (MRTK) cell contains intracytoplasmic whorled filamentous structures (x6,250); D, an NCR-W4 cell contains tightly packed filaments measuring 4–6 nm in diameter (x18,000).

were found in the heterotransplantable NBW line, OW1 (16). On the other hand, in MRTK line (CR-SW1), only one cytokeratin band with a molecular weight of 45,000, cytokeratin 18, was identified. No cytokeratin band was detectable in US line (CR-SW2).

DISCUSSION

SWTs were originally presented by Beckwith et al. as being possibly sarcomatous variants of Wilms' tumor because some of these, for example, contained epithelial elements differing from preexistent nephrons (1). However, by subsequent study, it had become apparent that these tumors should be viewed better as separate neoplastic entity from Wilms' tumors (2–4). The reason why we use the term SWT in this study is that their origins are entirely unknown and the exact relationship between SWT and NBW has not been made clear.

The histogenesis of NBW has well been documented including our own study (15). Our recent study clearly indicated that NBW contains a capability of differentiation comparable to that involved in fetal metanephric tissue, thus suggesting its metanephric origin. However, the histogenesis of SWT is still

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<td>Fetal kidney</td>
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<td>P, primary surgically resected tumors; C, cell lines heterotransplantable in nude mice or in vitro; P/C, both primary tumors and cell lines; B, blastemal elements; E, epithelial elements; S, stromal elements; M, metanephric blastemal cells; T, tubular structures of immature nephrons; G, immature glomeruli; S, stromal cells.</td>
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**HUMAN SARCOMATOUS WILMS' TUMOR LINES**

**Fig. 4.** Immunohistochemical staining of cytokeratin and vimentin on 23-week fetal kidney (A, B) and in vitro cell CCSK line, NCR-W1 (C, D). A, cytokeratin exists on tubular structures and its stainability is decreased during glomerular (G) differentiation from lower limbs of S-bodies (L) (× 220). B, vimentin exists on metanephric blastemas, stromas, and immature and mature glomeruli (G). Note coexpression of cytokeratin and vimentin (arrow) on lower limbs of S-bodies (L) (× 220). C, some CCSK cells are positive for cytokeratin (× 280). D, these cells are also positive for vimentin (× 280).

**Fig. 5.** Western blotting analysis of cytokeratin on SWT and NBW cells. Lanes 1, 3, 5, 7, 9, 11, anticytokeratin antibody MAK6; lanes 2, 4, 6, 8, 10, 12, anticytokeratin antibody K8.13. Cytokeratin 8 (M, 52,000) and 19 (M, 40,000) are identified in CCSK cell lines, both in vitro (lanes 1, 2, NCR-W1; lanes 3, 4, NCR-W3) and heterotransplantable (lanes 5, 6, CR-SW5). On the other hand, cytokeratin 18 (M, 45,000) is identified in heterotransplanted MRTK line (lanes 7, 8, CR-SW1) and no cytokeratin band is detected in heterotransplanted US line (lanes 9, 10, CR-SW2). Cytokeratins 8 and 19 are also identified in heterotransplanted NBW line (lanes 11, 12, OW).

The analyses on biological characteristics of SWT cells in vitro or in vivo have been restricted due to the limited number of available cell lines (22–24). Our study utilizing newly established cell lines, heterotransplantable or in vitro, could reveal more clearly some biological properties of SWT cells. Thus, there was significant heterogeneity in growth appearances between cell lines of CCSK and those of others. Most cell lines, especially MRTK and US, well maintained their original characteristics, consistent with the report of Vogel et al. (23) on MRTK. However, in CCSK lines, morphological changes often occurred, such as increase of cytoplasm-abundant cells and production of mucin. Ultrastructurally, clusters of intermediate filaments similar to those in MRTK cells, twisted sheaves of filaments resembling tonofilaments, and intracellular canaliculi could be identified in these cells. These results may represent very unique biological characteristics of CCSK cells. CCSK may be an elusive histopathological entity and it shows entirely controversial. The aim of this study is to establish biological property included in SWTs and to exploit their histogenesis.
a number of variations other than “classic pattern” (2). Furthermore, modification of histopathological features in the metastatic lesions was also reported (8, 14, 25). Gonzalez-Crussi noticed increased numbers of filaments, cilia, and other organelles in the metastatic tumors. Filaments filled the spaces between organelles, whereas in the primary tumors they were scanty and sparsely distributed (14). Such filament laden cells were sometimes found in non-MRTK renal tumors, which were described as “pseudo-rhabdoid” tumors of kidney (26). Metastatic lesions, made up almost entirely of myxomatous tissue, have also been reported (8, 25). Therefore, morphological changes found in our CCSK lines probably represented such variable histopathological feature of CCSK.

The most striking and interesting fact was that CCSK had a latent epithelial nature which became apparent in cell lines as revealed by the presence of twisted sheaves of filaments resembling tonofilaments and intracytoplasmic canaliculi. Schmidt et al. reported a case of CCSK containing true neoplastic epithelial elements (27), and Beckwith reevaluated the possibility of a closer relation between CCSK and NBW than previously suspected (28).

The epithelial nature of CCSK were clearly shown in this study by the expression of cytokeratins by immunoperoxidase and immunoblotting method. Primary tumors of CCSK were negative for cytokeratin, consistent with the previous reports (29, 30), whereas we could find the expression of cytokeratin on CCSK cell lines. Western blotting analysis demonstrated that CCSK expressed cytokeratin 8 (52KD) and 19 (40KD), the pattern identical to that found in NBW. From the literature, this cytokeratin pattern is related to simple epithelium (31). Recently, it was reported that nonepithelial tissue such as myometrium and fetal myocardium expressed cytokeratins 8, 18, or 19 (32, 33). However these cells did not show any morphological epithelial nature, whereas newly established CCSK lines apparently showed morphologically epithelial features as described. Therefore, the identical expression of cytokeratins 8 and 19 found in both CCSK and NBW indicated that there was close relationship between CCSK and NBW. MRTK, on the other hand, expressed different cytokeratin from that found in CCSK and NBW, which became evident by Western blotting analysis. In addition, MRTK had ultrastructurally no epithelial feature. Taking these results into account, there arises a possibility that MRTK belongs to another neoplastic entity from Wilms' tumor. Finally, US seems to be a very primitive neoplasm without any features of CCSK and MRTK, because no alteration in morphology and cytokeratin expression has been observed in US even in the cell lines.

Analysis on the expression of cytokeratin, vimentin, and CD24 in fetal kidney presented an interesting result for the histogenesis of CCSK. In the fetal kidneys, coexpression of cytokeratin and vimentin was demonstrated at different stages: the tubular formation stage from the metanephric blastema and the glomerular differentiation stage from the lower limbs of S-bodies (34). Interestingly, CD24 can distinguish these two embryologically different stages. Thus, metanephric blastemases were positive for CD24 but lower limbs were not. Assuming that tumor cells conserve the phenotypes of normal counterpart, CCSK may originate from lower limbs of S-bodies or closely related tissues because phenotypes of CCSK cell lines (cytokeratin", CD24") correspond to these tissues. Although further study is clearly necessary to establish this hypothesis, it is of note to introduce a monoclonal antibody 5H10 which we have established against SWT. Preliminary experiments suggest that 5H10 reacts with the molecule expressed on cell surface of CCSK as well as on lower limbs part of S-bodies showing stage-specific fashion. Precise study of 5H10 is now under investigation.

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REFERENCES


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